Biochemical Studies on the Effect of Low and Excess Biotin Intakes on Female and Male Reproduction in Rodents

2014

Hiromi Sawamura

Table of Contents

Abstract

Biotin is a water-soluble vitamin that is classified as a B-group vitamin. In mammals, it serves as an essential cofactor for four carboxylases in fatty acid synthesis, branched-chain amino acid metabolism and gluconeogenesis. The three main purposes of this study are: (1) to clarify the effects of maternal biotin deficiency on biotin homeostasis in both dams and fetuses, (2) to clarify the effects of the excess intake of biotin on early growth and (3) to clarify the effects of the excess intake of biotin on spermatogenesis.

As described in chapter 2, to clarify the effects of maternal biotin deficiency on biotin homeostasis in mammals, we examined whether low maternal biotin intake affects the expression of any gene that plays an important role in maintaining biotin homeostasis in mice. Pregnant mice were fed a biotin-deficient diet or a biotin-supplemented (control) diet for 14 days of gestation. The incidence of cleft palate among fetuses in the biotin-deficient group was 97.4%. The biotin concentration was significantly decreased in all tissues examined, except maternal kidney of biotin-deficient mice, compared with that in the control. In the placenta, the ratios of sodium-dependent multivitamin transporter (SMVT) mRNA and protein expression in the biotin-deficient group were significantly higher than those in the control. However, the expression of holocarboxylase synthetase and biotinidase mRNA was not significantly different between the two dietary groups. We confirmed that low maternal biotin intake changes the expression of SMVT *in vivo*. These

results demonstrate that maternal biotin deficiency may be associated with biotin transporter upregulation accompanied by the abnormal development of fetuses.

As described in chapter 3, to clarify the effects of excess intake of biotin on early growth, weaning rats were fed on a 20% casein diet containing 0.00002% biotin, or the same diet with 0.04%, 0.08%, 0.1%, 0.2%, 0.5%, 0.8% or 1.0% added biotin for 28 days. The administration of more than 0.08% biotin decreased the food intake and body weight gain compared with the levels in control rats. The accumulation of biotin in such tissues as the liver, brain and kidney increased in a dose-dependent manner. We confirmed that excess intake of biotin adversely affects early growth. In addition, this study showed that testis weights in the 1.0% biotin group were significantly lower than those in the control. This indicates that excess intake of biotin may particularly affect testicular function.

Next, we focused on the effects of the excess intake of biotin on male reproduction. As described in chapter 4, to clarify such effects in mammals, the effects of high dietary doses of biotin on spermatogenesis were biochemically and histologically investigated in male rats. Weaned rats were fed a control diet containing 0.00004% biotin, or a control diet supplemented with 0.01%, 0.1% or 1.0% biotin. Pair-fed rats were fed a control diet that was equal in calories to the amount ingested by the 1.0% biotin group because food intake was decreased in the 1.0% biotin group. Food intake and body weight gain were lower in the 1.0% biotin group than in the control group. The kidney, brain and testis weights were significantly lower in the 1.0% biotin group than in the

pair-fed group after 6 weeks of feeding. The accumulation of biotin in the liver and testis increased in a dose-dependent manner. In the 1.0% biotin group, the number of mature sperm was markedly lower, while that of sperm with morphologically abnormal heads, mainly consisting of round heads, had increased. In addition, the development of seminiferous tubules was inhibited, and few spermatogonia and no spermatocytes were histologically observed. These results demonstrate that the long-term intake of excess biotin inhibited spermatogenesis in young male rats.

In conclusion, we first demonstrated that low maternal biotin intake changes the expression of SMVT and the excess intake of biotin inhibits spermatogenesis. We confirmed that biotin is essential for reproduction and development in both males and females, but its excess intake may adversely affect the reproductive system. Further study is needed to determine the recommended intake of biotin for pregnant women and to set a tolerable upper intake level for biotin.

Chapter 1. General Introduction

Vitamins are essential for growth and development. The influence of various vitamins on female and male reproduction has been studied by several research groups. For example, in terms of deficiency in fat-soluble vitamins, it has been demonstrated that vitamin D deficiency resulted in low reproductive capacity[1]. Wang et al. reported the effects of vitamin E on sperm functions in spinal cord-injured rats[2]. The importance of vitamin A in male reproduction was also discovered[3] and a recent study suggested that a vitamin A metabolite, retinoic acid, is needed for adult male spermatogonial differentiation[4]. On the other hand, excess vitamin A levels in rats were shown to reduce the weight of the testes and to enlarge the nuclei of spermatocytes[5].

With regard to water-soluble vitamins, vitamin B_{12} deficiency was shown to cause increments of abnormal and dead sperm[6]. Vitamin C deficiency also disturbs spermatogenesis; therefore, the dietary prescription of vitamin C has been shown to protect germ cells from oxidative stress throughout spermatogenesis in humans[7]. A low level of folate in seminal plasma is correlated with decreased sperm count and increased sperm DNA damage in humans[8,9]. In addition, a recent study demonstrated that pantothenic acid is an essential factor in testicular endocrinology and sperm motility in male rats[10]. On the other hand, mega-doses of vitamin B_6 (pyridoxine) affected spermatogenesis and decreased reproductive organ weights in rats[11]. A number of studies have reported on the effects of vitamin deficiencies on the male reproductive system, but only a few have looked at the effect of excess vitamin intake.

Numerous studies have been reported concerning the role of vitamins in female reproduction. Several studies demonstrated that maternal vitamin D deficiency affected fetal development through an effect on maternal calcium homeostasis[12-14]. Scholl et al. suggested that vitamin E (α-tocopherol) is positively associated with fetal growth by promoting greater blood flow and nutrient supply to the fetus[15]. In addition, vitamin A deficiency has been shown to lead to fetal resorption or malformation[16].

The association of low maternal vitamin B_{12} concentration with impaired fetal development has been demonstrated[17]. Folate deficiency interferes with growth of the conceptus, uterus and placenta through the effects on DNA synthesis and cell replication[18]. Pantothenic acid deficiency in female mice leads to abnormal development of the ovary and uterus[19]. Roecklein et al. suggested that vitamin B_1 (thiamine) deficiency during in utero development may contribute to intrauterine growth retardation[20]. Several rodent studies have shown that vitamin B_2 (riboflavin) deficiency increases the incidence of congenital malformations[21,22]. Alton-Mackey et al. showed that infant rats born to vitamin B6-deprived dams had lower body weight and impaired neuromotor development[23]. In the present study, we focused on the effect of biotin on female and male reproduction.

Biotin is a water-soluble vitamin that is classified as a B-group vitamin. Boas first demonstrated the requirement for it in mammals over 80 years ago[24]. In mammals, biotin is covalently bound to pyruvate carboxylase (PC), acetyl-CoA carboxylase (ACC), propionyl-COA

carboxylase (PCC) and 3-methylcrotonyl-CoA carboxylase (MCC)[25,26]. Holocarboxylase synthetase (HCS) catalyzes the covalent attachment of biotin to these biotin-dependent carboxylases[27]. Biotin serves as an essential cofactor for four carboxylases in fatty acid synthesis, branched-chain amino acid metabolism and gluconeogenesis[28]. Therefore, biotin deficiencies cause the dysfunction of these metabolic pathways, and the resulting biochemical and physiological impairments induce skin disorders such as dermatitis, hair loss, neuritis and susceptibility to infections[28]. A recent study showed that biotin also plays roles in the epigenetic regulation of genes[29]. Kothapalli et al. identified a novel post-translational modification: covalent binding of biotin to lysine residues in histones[30].

Mammals cannot synthesize biotin and must depend on dietary intake from microbial and plant sources or microbes in the intestines[31,32]. Biotin exits in food as free and protein-bound forms[33]. Biotinidase catalyzes the cleavage of biotin from biocytin (biotinyl-lysine) or short biotinylated peptides[34], thereby recycling biotin. Therefore, biotinidase deficiency causes metabolic disorder of biotin recycling. In mammals, the sodium-dependent multivitamin transporter (SMVT) transports some water-soluble vitamins, biotin, pantothenate and liponate[35]. SMVT is expressed in various tissues, such as placenta, intestine, liver and kidney[36]. Biotin deficiencies are rare in humans, as biotin is well distributed in various kinds of food. However, biotin deficiency can be induced by consuming large amounts of raw egg whites, containing avidin.

Numerous studies have shown that biotin is essential for reproduction and embryonic development in mammals[37-40]. Additionally, it has recently been demonstrated that decreased urinary excretion of biotin in the late stage of gestation is observed even in normal pregnancy, suggesting that pregnant women may experience mild biotin deficiency[41,42]. However, the relationship between biotin and fetal development is not well known. As described in chapter 2, the aim of one part of this work was to clarify the effects of biotin deficiency during pregnancy on maternal and fetal biotin homeostasis through the regulation of biotin transfer in mammals. HCS, BTD and SMVT play crucial roles in biotin homeostasis by regulating biotin absorption and recycling in mammals[43]. Therefore, whether low intake of biotin during pregnancy affects the expression of these three proteins in maternal and fetal tissues in mice was examined.

Regarding the excess intake of biotin, the 2010 version of the Dietary Reference Intakes for Japanese presented no data on its tolerable upper intake level (UL)[44]. The risks associated with the excess intake of biotin have not yet been established in healthy humans. In patients with holocarboxylase synthetase and biotinidase deficiencies, the administration of oral biotin at doses up to 100 mg/day did not result in adverse effects[45]. In rodents, the oral LD_{50} of biotin was reported to be >10 g/kg in mice[46] and >354 mg/kg in rats[47]; however, in these studies, the details were not provided. Therefore, as described in Chapter 3, the effects of excess orally administered biotin (0.01% to 1.0% biotin/kg diet) on early growth in young male rats were investigated.

In studies on the effects of excess biotin intake on development and reproduction, Mitthelholzer suggested that biotin at a dose totaling 50 mg/kg b.w. by subcutaneous injection did not affect reproduction in female rats[48]. Watanabe also reported that the oral administration of 1 g biotin/kg diet to pregnant mice during gestation had no effect on the rate of successful pregnancies[49]. However, it remains unknown whether excessive amounts of biotin can affect reproduction in male animals. As described in chapter 4, focusing on the effects of the excess intake of biotin on male reproduction, the biochemical and histological effects of excess biotin (0.01% to 1.0%) intake on cell growth and sperm maturation during spermatogenesis were examined.

Chapter 2. Low Maternal Biotin Intake Changes the Expression of Biotin Transporter in Mice

2.1 Introduction

It is known that biotin deficiency causes the dysfunction of these metabolic pathways, and the resulting biochemical and physiological impairments induce skin disorders such as dermatitis, hair loss, neuritis and susceptibility to infections[28]. Biotin deficiencies are rare in humans, as biotin is well distributed in various kinds of food. However, biotin deficiency can be induced by consuming large amounts of raw egg whites, which contain high levels of avidin. Avidin is known to inhibit the absorption of biotin from the intestinal tract and to produce biotin deficiency[28]. It is also reported that biotin deficiency is induced in patients provided anticonvulsants[50] and in infants fed with special therapeutic infant formulas in Japan[51].

Biotin is essential for reproduction and fetal development in mammals. We first detected that maternal biotin deficiency causes severe malformations in mouse fetuses[37-38]. The external malformations are mainly cleft palate, micrognathia and micromelia in the ICR and A/Jax strains. We previously detected that there are strain and species differences in the teratogenic effects of biotin deficiency in rodents[39-40]. It has been demonstrated that decreased urinary excretion of biotin in the late stage of gestation is observed even in normal pregnancy, suggesting that pregnant women may suffer mild biotin deficiency[41-42]. In pregnant mice, biotin excretion in urine decreased on day

of gestation (dg) 4 in biotin-deficient dams and on dg 16 in biotin-supplemented dams[52-53]. The requirement for biotin may increase during gestation and/or fetal development at the specific stages. A large amount of biotin compared to the normal stage is necessary for maintaining normal reproductive performance during the late stage of gestation. The relationship between biotin and fetal development is not well known.

The present study aimed to clarify the effects of biotin deficiency during pregnancy on three genes related to biotin homeostasis in mammals. Sodium-dependent multivitamin transporter (SMVT), holocarboxylase synthetase (HCS) and biotinidase (BTD) play crucial roles in biotin homeostasis by regulating biotin absorption and recycling[43]. SMVT, which transports some water-soluble vitamins, biotin, pantothenate and liponate, is expressed in various tissues such as placenta, intestine, liver and kidney[35-36]. Ghosal et al. reported that conditional knockout of the SMVT gene in mouse intestine caused growth retardation and decreased bone density and length[54]. There are no reports about the effect of biotin deficiency on SMVT expression *in vivo*. Meanwhile, HCS catalyzes the biotinylation of carboxylases[55] and histones[56], and BTD is the enzyme responsible for the recycling of biotin, the transport of biotin in plasma[57] and the regulation of histone biotinylation[58]. In humans, the biotin cycle was shown to be disrupted by genetic deficiency of HCS or BTD[59], but it remains unclear whether these three proteins directly affect biotin metabolism during pregnancy. Therefore, we examined whether a low intake of biotin during pregnancy affects the expression of these proteins in maternal and fetal tissues in mice.

2.2 Materials and methods

2.2.1 Animals and diet

Nulliparous female ICR mice, aged 6 weeks, were obtained from CLEA Japan Inc. (Tokyo, Japan). All animals, including males used for mating, were housed for 2 weeks before mating in an animal room maintained under 12 h light-dark cycle conditions of 0900-2100 and at a constant room temperature of 23 ± 2 °C. The female mice were mated with healthy males for a short mating period in the morning (0900-1100). The day when a copulation plug was detected at the end of mating was designated as day 0 of gestation (dg 0). Pregnant females were randomly divided into two groups: a biotin-deficient group (n=11) fed a biotin-deficient diet (Table 2-1) and a control group (n=10) fed a biotin-supplemented diet (biotin-deficient diet supplemented with 5 mg biotin/kg). These mice were housed in stainless steel cages with a wire-bottomed floor and given the diets and distilled water *ad libitum* for 14 days (full term = 19 days). Diet consumption had been confirmed to be approximately the same in the two dietary groups in our previous study[4]. All experimental procedures including the care and treatment of mice described in this paper were approved by the Institutional Animal Care and Use Committee of the School of Human Science and Environment, University of Hyogo (#038, 087).

2.2.2 Collecting samples

Pregnant mice were killed on dg 14 because, in normal murine craniofacial development, the secondary palates undergo major organogenesis on dg 12-15[60]. Serum was collected to measure biotin concentration and biotinidase activity, and was stored at -20 °C until needed. Fetuses were collected from the uterus and immersed in phosphate-buffered saline (PBS). The placenta was removed from the fetuses and the number of fetuses was confirmed. Maternal tissues (brain, liver, kidney and placenta) and fetal tissues (liver and palatal process) were collected. Palatal processes were carefully dissected from the head in fetuses under a dissecting microscope using a technique described previously[21]. These samples were immediately stored at -80 °C until analysis.

2.2.3 Measurements of biotin concentration and biotinidase activity

Blood was centrifuged for 10 min at 3,000 rpm and serum was collected. Tissues were lysed with solubilization buffer (1% Triton-X100 and 0.02% protease inhibitor in PBS). These tissue samples were homogenized on ice using a sonicator. The sonicated samples were centrifuged at 15,000 rpm for 15 min at 4 °C and the supernatant was collected.

Biotin concentration in tissues was determined using a microtiter plate adaptation of a microbiological assay with *Lactobacillus plantarum* ATCC 8014[61-63]. This bacterium was obtained from American Type Culture Collection, which is generally used for determining the quantity of some vitamins, cultured in a microtiter plate for 24 h and the cell density was determined at 610 nm. As biotin in tissues partially existed in a protein-binding form, for the determination of total

biotin, 100 μL of sample solution was pretreated with 2.25 M H_2SO_4 for 121 °C for 60 min and neutralized with 4.5 M NaOH. Biotin concentrations are expressed as pmol/mL or nmol/g protein. The protein concentration of samples was determined with a BCA Assay kit (Thermo Fisher Scientific Inc., Kanagawa, Japan). Biotinidase activity was measured using the colorimetric method by measuring the liberation of *p*-aminobenzoate from *N*-biotinyl-*p*-aminobenzoate. Biotinidase activity is expressed as nmol/min/mL or nmol/min/g protein.

2.2.4 Quantitative real-time PCR

Total RNA from tissues was isolated using TRIzol reagent (Life Technologies Japan Ltd., Tokyo, Japan), and complementary DNA was synthesized using a ReverTra Ace qPCR RT Master Mix (TOYOBO Co. Ltd., Osaka, Japan). Quantitative mRNA expression was assessed via SYBR Green qPCR assay. The gene-specific primer sequences were as follows: for SMVT, forward 5'-ACGCAAGGCAAGCAGAAC-3' and reverse 5'-GCACCGACTGATTCTGTGAGTA-3'; for HCS, forward 5'-TCCAGCATTTGATGTCCTTG-3' and reverse 5'-TATCGTTGGGCCACTTCACT-3'; for BTD, forward 5'-CATCCATCGGTCCTGAGC-3' and reverse 5'-TAATCTGCACACCCTTCTGG-3'; for β-actin, forward 5'-CTAAGGCCAACCGTGAAAAG-3' and reverse 5'-ACCAGAGGCATACAGGGACA-3'. The mRNA levels were assessed with β-actin as an internal control under the following conditions: pre-incubation at 98 °C for 2 min, followed by 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 68 °C for 30 s. All qPCR was performed in KOD

SYBR qPCR mix (TOYOBO Co. Ltd., Osaka, Japan) on a StepOne Real-time PCR System (Applied Biosystems Inc., Japan, Tokyo, Japan). The results were normalized to β-actin. Fold change expression was calculated using threshold cycle (Ct) values and determined via the $2^{-\Delta\Delta CT}$ method[64].

2.2.5 Western blot analysis

Tissues samples were homogenized as described above. The protein concentration of samples was determined with a BCA Assay kit (Thermo Fisher Scientific Inc., Kanagawa, Japan). Each sample was adjusted to the same concentration of protein and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Extracted protein was heated at 100 °C for 5 min before loading. Samples were then separated on 8% gels, transferred onto polyvinylidene difluoride (PVDF) membranes (Pall Fluoro Trans W membrane, NIPPON Genetics Co., Ltd., Tokyo, Japan) and blocked for 1 h at room temperature in 1% bovine serum albumin. Monoclonal anti-β-actin (1:3000; Sigma-Aldrich Co. Ltd., Tokyo, Japan) and polyclonal anti-SMVT (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, USA) were obtained to detect these antigens. Membranes were incubated with these antibodies overnight at 4 °C and were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature. Target protein was detected using the enhanced chemiluminescence system (GE Healthcare Japan Co., Ltd., Tokyo, Japan).

2.2.6 Statistical analysis

The values in the text are expressed as mean ± SD. To assess the effects of biotin deficiency, statistical comparison of means of two experimental groups was conducted by Student's *t*-tests. Statistical analysis of the data was performed on a personal computer using a standard statistical package (Statcel Ver. 3, Tokyo, Japan). Differences were considered statistically significant if *P* values less than 0.05 in all analyses.

2.3 Results

2.3.1 Body weight and growth

No significant differences were observed between the two dietary groups for food intake, body weight gain and fetal number (data not shown). These results are consistent with our previous studies of the effect of biotin deficiency during pregnancy on mice[65]. No clinical signs of biotin deficiency in dams were observed. Maternal tissue weights were also not significantly different between the two dietary groups (Table 2-2). Incidence of cleft palate of fetuses in the biotin-deficient group was 97.4%.

2.3.2 Biotin concentration and biotinidase activity

The biotin contents in the tissues are shown in Table 2-3. In biotin-deficient mice, total biotin concentration was significantly decreased in the maternal serum (22% of control), liver (59%),

placenta (16%), fetal liver (3%) and palatal process (10%) compared with the control group. In particular, the biotin contents were markedly decreased in maternal serum and fetal liver. These results consistent with our previous study[65]. Meanwhile, in maternal kidney, biotin contents did not differ between the two dietary groups. The ratio of free biotin was significantly increased in the maternal liver of biotin-deficient mice (125% of control), while a significant decrease was observed in fetal palatal process (26%). Biotinidase activity showed no significant difference in these tissues between the two dietary groups (Table 2-4).

2.3.3 SMVT, HCS and BTD gene expression

In terms of the expression of mRNA in the tissues, quantitative RT-PCR analysis showed that the relative level of SMVT mRNA was significantly increased in the placenta and fetal liver of the biotin-deficient group compared with the control group (Fig. 2-1A). Meanwhile, no significant differences were observed in maternal liver and fetal palatal process. The expression of HCS and BTD mRNA was not significantly different in all tissues examined between the two dietary groups (Fig. 2-1B, C).

2.3.4 Biotin transporter protein expression

Western blot analysis demonstrated that the expression of SMVT protein was significantly elevated in the placenta of biotin-deficient mice compared with control group (Fig. 2-2B). In

maternal liver, SMVT protein expression showed a pattern to decrease in biotin-deficient mice (p=0.100) (Fig. 2-2A). There was no significant difference in fetal liver between the two dietary groups (Fig. 2-2C).

2.4 Discussion

We suggested in previous studies that biotin deficiency during pregnancy in mice causes a remarkably high incidence of congenital malformations such as cleft palate, micromelia and micrognathia in fetuses[37-38,66-67]. Levin et al. also suggested that rat fetuses from dams given a biotin-deficient diet throughout gestation had some obvious dysmorphic features[68]. These studies suggested that biotin may be required to maintain normal pregnancy and fetal development in the middle stage of gestation. However, it remains unclear how maternal biotin deficiency affects biotin homeostasis during pregnancy through the regulation of biotin transfer. In order to clarify the effect of maternal biotin deficiency on the maintenance of biotin homeostasis during pregnancy, we studied the expression of three genes related to biotin homeostasis in mammals.

SMVT (product of the *SLC5A6* gene) is essential for mediating and regulating biotin uptake into mammalian cells. In the present study, we showed that mRNA levels of biotin transporter SMVT were increased in accordance with biotin deficiency during pregnancy in the placenta and fetal liver. SMVT protein expression was also increased in the placenta, but there was no significant difference in the fetal liver. Meanwhile, in maternal liver, mRNA levels of SMVT did not differ

between the two dietary groups, but SMVT protein expression showed a pattern of decrease in biotin-deficient mice. Absorptive tissues, such as the intestinal mucosa and kidney, and the placenta have very high levels of SMVT-specific mRNA[69]. The placenta plays a key role in the maternal-fetal relationship, maintaining fetal homeostasis through the regulation of nutrient transfer. It has been reported that biotin deficiency decreased the level of hSMVT in human liver HepG2 cells[70] and leukocytes[71]. On the other hand, Reidling et al. demonstrated that biotin deficiency leads to an increase in the protein and mRNA levels of hSMVT in human intestinal epithelial cells[72]. In addition, Crisp et al. suggested that biotin concentration correlates negatively with the expression of SMVT in human choriocarcinoma cells[73]. These studies indicated that the effects of biotin deficiency on SMVT expression might differ between tissues that have high levels of SMVT, such as intestine and placenta, and other tissues. We suggested that maternal biotin deficiency leads to changes in the expression of SMVT in the placenta in an attempt to maintain the homeostasis of biotin.

It was demonstrated that maternal folate deficiency is associated with folate receptor upregulation accompanied by multiple aberrations in fetal tissues[74]. Kim et al. found that paternal folate deficiency affected folate transport in the placenta[75]. These studies suggested that folate deficiency reduced the folate content with an increase in the expression of folate receptor in the placenta. The mechanism of these seemingly contradictory findings is unclear. The role of placenta has recently received great attention because of its importance in epigenetic regulation during the development of offspring[76]. Biotin is covalently attached to histones and biotinylation of histones

plays a role in cell proliferation, gene silencing and cellular response to DNA damage[30]. Maternal biotin deficiency might affect fetal development through the impact on placental development and function.

In the present study, the expression of SMVT mRNA was increased in accordance with biotin deficiency in the fetal liver, whereas its protein expression was not modified. The protein expression of SMVT was not consistent with mRNA expression, implying the existence of post-transcriptional regulation of SMVT. MicroRNAs (miRNAs) are a class of small non-coding functional RNAs that mediate post-transcriptional regulation of gene expression[77]. miRNAs reduce the translation and/or stability of that mRNA, leading to a reduction in protein levels. A recent study showed that the expression of miR-539 depends on biotin's regulation of the expression of HCS in human kidney cells[78]. miRNA may play roles in the regulation of SMVT expression.

HCS and *BTD* gene expression in maternal liver, placenta, fetal liver and palatal process was unchanged by biotin deficiency in the present study. Biotinidase activity in these tissues also did not differ between the two dietary groups. Rodríguez-Meléndez et al. suggested that HCS mRNA was significantly decreased in the liver, kidney, muscle and brain in biotin-deficient male rats[79]. A recent study showed that HCS acts as a biotin sensor, which may be involved in post-translational regulation of SMVT expression in Jurkat cells[80]. On the other hand, it has been demonstrated that hepatic mRNA for HCS did not change significantly in either dams or fetuses in mice[81], which is consistent with our findings. These studies indicate that the effects of biotin deficiency on HCS

expression may be specific to the gender and the type of cell. In terms of BTD expression, gene expression was not affected by biotin supply in human choriocarcinoma cells[73]. We suggested that the biotin recycling system does not act to maintain the homeostasis of biotin when dams become biotin-deficient.

In conclusion, we detected that a low intake of biotin during pregnancy changes SMVT gene and protein expression. There are no reports about the effect of biotin deficiency on SMVT expression *in vivo*. We first confirmed that a low level of maternal biotin intake changes the expression of SMVT. These results demonstrated that maternal biotin deficiency may be associated with biotin transporter upregulation accompanied by abnormal development of the fetuses. Further studies about the relationship between maternal biotin deficiency and fetal development are needed.

Table 2-1 Component of the biotin-deficient diet

Ingredient	Amount (%)
Egg white, spray dried	20
L-cystine	0.3
Corn starch	39.7486
a-Corn starch	13.2
Sucrose	10
Soybean oil	7
Cellulose powder	5
Mineral mix (AIN-93G)	3.5
Biotin-free vitamin mix (AIN-93G)†	1
Choline bitartrate	0.25
tert-Butylhydroquinone	0.0014

† Component of the biotin-free vitamin mix

Vitamin A (All-trans-retinyl paimitate) [500,000U/g]	0.08
Vitamin D_3 (Cholecalciferol) [400,000IU/g]	0.025
Vitamin E (All-rac-a-Tocopheryl Acetate) [50%]	1.5
Vitamin K_1 (Phylloquinon)	0.0075
Vitamin B_1 (Thiamine hydrochloride)	0.06
Vitamin B_2 (Riboflavin)	0.06
Vitamin B_6 (Pyridoxine Hydrochloride)	0.07
Vitamin B_{12} (Cyanocobalamin) [0.1%]	0.25
Folic acid	0.02
Calcium pantothenate	0.16
Nicotinic acid	0.3
Sucrose	97.4675

Table 2-2 Effects of maternal biotin deficiency on maternal tissue weights.

	Brain	Liver	Kidney	Placenta
Control	0.49 ± 0.05	2.73 ± 0.18	0.24 ± 0.01	0.08 ± 0.01
Biotin-deficient	0.47 ± 0.02	2.36 ± 0.27	0.22 ± 0.01	0.08 ± 0.01

Each value is expressed as the mean ±SD (n=3).

Table 2-3 Biotin concentration in maternal and fetal tissues.

			Dietary groups	
			Control	Biotin-deficient
Dams	Serum	total biotin (pmol/mL)	132.9 ± 53.3	29.2 ± 11.2**
		free biotin (pmol/mL)	109.8 ± 18.4	14.6 ± 0.9**
		free to total ratio (%)	54.4 ± 10.2	53.8 ± 19.3
	Liver	total biotin (nmol/g of protein)	26.4 ± 5.8	15.5 ± 2.3*
		free biotin (nmol/g of protein)	3.5 ± 0.3	2.6 ± 0.2*
		free to total ratio (%)	13.5 ± 1.7	16.9 ± 1.2*
	Kidney	total biotin (nmol/g of protein)	27.6 ± 8.1	26.1 ± 2.4
		free biotin (nmol/g of protein)	1.0 ± 0.3	0.7 ± 0.0
		free to total ratio (%)	4.0 ± 2.5	2.6 ± 0.3
	Placenta	total biotin (nmol/g of protein)	11.8 ± 8.1	1.9 ± 0.4*
		free biotin (nmol/g of protein)	4.6 ± 1.8	0.4 ± 0.1*
		free to total ratio (%)	40.5 ± 15.5	24.4 ± 8.9
Fetuses	Liver	total biotin (nmol/g of protein)	86.1 ± 25.4	2.3 ± 0.5**
		free biotin (nmol/g of protein)	72.5 ± 18.7	1.3 ± 0.2**
		free to total ratio (%)	86.3 ± 16.2	57.2 ± 8.6
	Palatal process	total biotin (nmol/g of protein)	23.0 ± 20.6	2.3 ± 1.0
		free biotin (nmol/g of protein)	14.4 ± 10.1	0.5 ± 0.4
		free to total ratio (%)	73.1 ± 17.5	18.8 ± 11.3*

Each value is expressed as the mean ±SD (n=3-11).

*$P < 0.05$, **$P < 0.01$, compared with the control group.

Table 2-4 Biotinidase activity in maternal and fetal tissues.

		Dietary groups	
		Control	Biotin-deficient
Dams			
	Serum (nmol/min/mL)	7.3 ± 1.3	7.9 ± 1.4
	Liver (nmol/min/g protein)	357.9 ± 109.9	378.1 ± 73.9
	Kidney (nmol/min/g protein)	508.6 ± 44.8	541.2 ± 68.1
	Placenta (nmol/min/g protein)	164.3 ± 61.0	198.7 ± 95.0
Fetuses			
	Liver (nmol/min/g protein)	76.1 ± 31.7	62.6 ± 39.9
	Palatal process (nmol/min/g protein)	117.5 ± 66.2	191.5 ± 118.1

Each value is expressed as the mean ±SD (n=3).

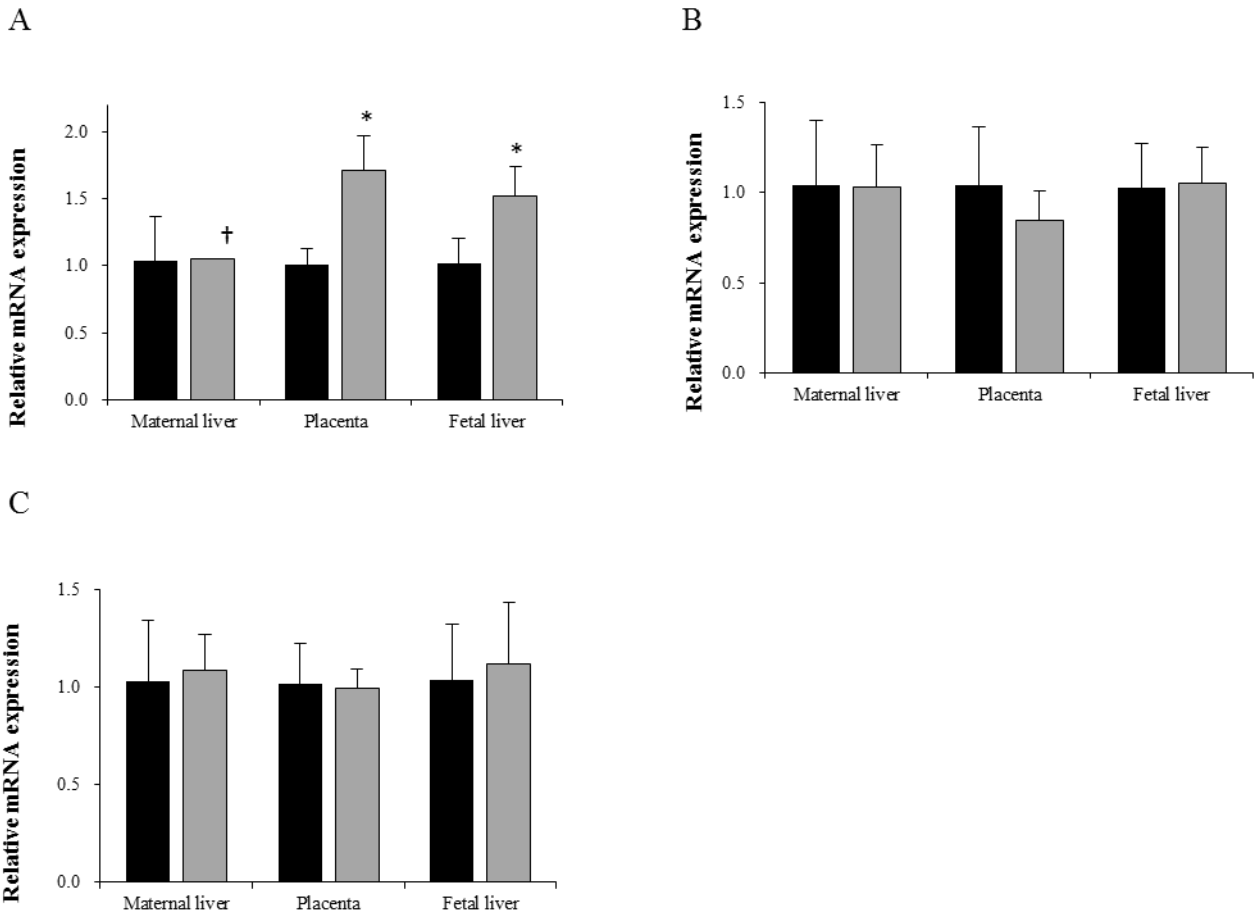

Fig. 2-1 Effects of biotin deficiency on gene expression of SMVT (A), HCS (B) and BTD (C) in maternal and fetal tissues. Ct value were normalized with β-actin as a housekeeping gene. Relative expression of mRNA is represented as fold change in comparison to the control group. Black bar, control; gray bar, biotin-deficient. Each value is expressed as the mean ± SD (n=3-6). *P < 0.01, compared with the control group.

†One value of the data of SMVT mRNA expression in the maternal liver of the biotin-deficient group was deleted since this value was unusually high (about 5 times higher than in the control group). When this value is included, the mean value becomes 2.47 ± 2.44 (n=3).

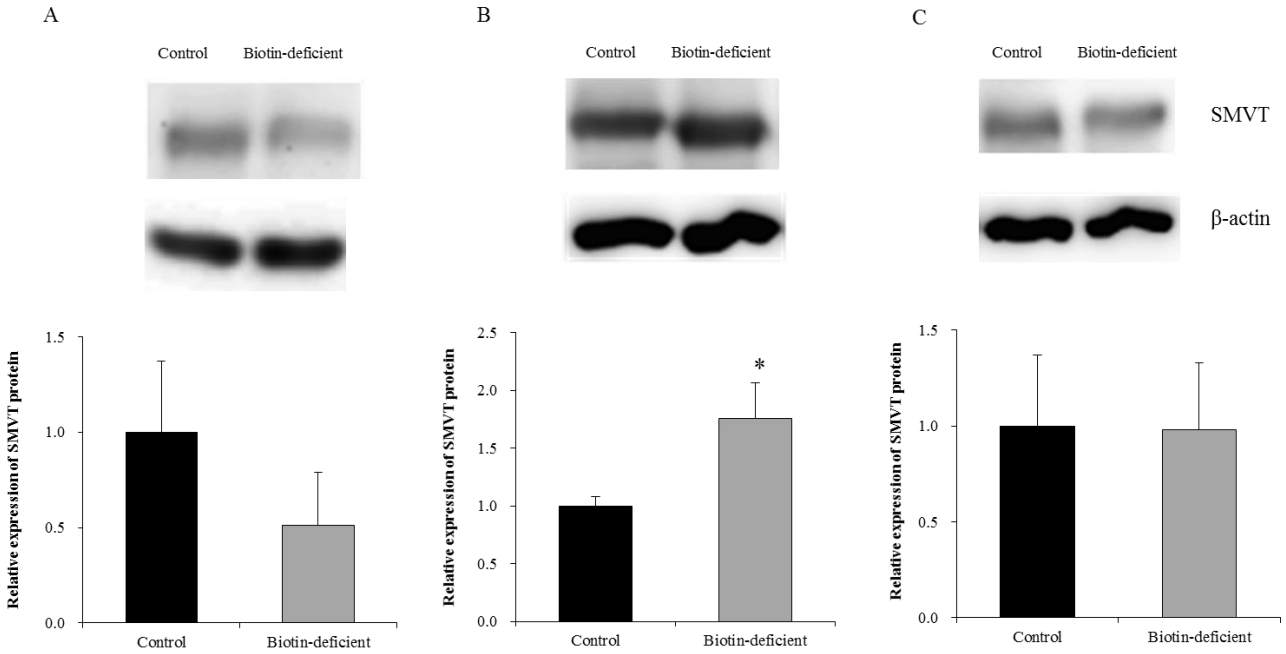

Fig. 2-2 Effects of biotin deficiency on SMVT protein expression in maternal and fetal tissues: (A) maternal liver, (B) placenta, (C) fetal liver. SMVT protein in tissues was detected by Western blot analysis. A 69-kDa band corresponding to SMVT protein was detected. The intensity of individual bands was quantified using Image J densitometry software**.** As an internal control, β-actin (42 kDa) was used for normalization**.** The ratio for the control group was assigned a value of 1. The results for one representative sample and the normalized average values for the 3 samples studied, compared with the control group, are shown. Each value is expressed as the mean ± SD (n=3). **P* < 0.05, compared with the control group.

Chapter 3. Effects of Excess Intake of Biotin on the Growth in Young Rats

3.1 Introduction

Biotin is a heterocyclic compound, an imidazolidone ring joined to a tetrahydrothiophene ring. The latter possesses a valeric acid side chain. The structure is unique, and biotin is more toxic than would be expected if a repeated excess dosage is administered. Some people have recently been taking 1-10 mg/d of biotin as a medical treatment because biotin has been found to be correlated with certain diseases such as diabetes mellitus[82,83], liver[84] and skin[85] disorders, neurological abnormality[86] and epilepsy[87].

Single or repeated doses of biotin (total doses of 50 and 100 mg/kg body weight by subcutaneous injection) given to rats resulted in irregularities of the estrus cycle[88,89] and fetal and placental resorption in pregnant rats[88], accompanied by decreased uterine weight, reduced glycogen and protein in the uterus, and reduced protein in the liver. However, these studies cannot be regarded as conclusive for human dietary biotin uptake, because of the route of administration.

The administration of oral biotin in doses up to 100 mg/day to patients with holocarboxylase synthetase and biotinidase deficiency has not resulted in adverse effects[45], although the metabolic defect may prevent or mask toxicity. The Japanese Dietary Reference Intake recommendation presents no data on the tolerable upper intake level (UL) for biotin[44]. Biotin toxicity in healthy humans has not been studied, and performing such a study with the risk of an adverse effect would

not be permitted. In the present study, we investigated the effects of excess orally administered biotin on the food intake, body weight gain, organ weight in young rats.

3.2 Materials and methods

3.2.1 Chemicals

Vitamin-free milk casein, sucrose, and L-methionine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Corn oil was purchased from Ajinomoto (Tokyo, Japan). Gelatinized corn starch, the mineral mixture (AIN-93M)[90] and vitamin mixture (AIN-93-VX containing 25% choline bitartrate)[90] were obtained from Oriental Yeast (Tokyo, Japan). D(+)-biotin ($C_{10}H_{16}N_2O_3S$, 244.31) were purchased from Wako Pure Chemical Industries. All other chemicals used were of the highest purity available from commercial sources.

3.2.2 Animals

The care and treatment of the experimental animals conformed to the University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals.

Male Wistar rats (3 weeks old) were obtained from CLEA Japan (Tokyo, Japan) and placed in individual metabolic cages (CT-10; CLEA). They were divided into eight groups (such group consisting of four rats) and fed *ad libitum* for 28 days: one group with a 20% casein diet (used as a

control group containing 0.000002% biotin), and the others with the same diet plus 0.04, 0.08, 0.10, 0.20, 0.50, 0.80 or 1.0% biotin (Table 3-1).

The room temperature was maintained at around 22°C and 60% humidity, and a 12 h light (06:00–18:00)/12 h dark (18:00–06:00) cycle was maintained. The body weight and food intake were measured every 2 days at around at 10:00. Urine samples (24 h; 10:00–10:00) were collected in amber bottles containing 1mL of 1 M HCl on the last day of the experiment, and were stored at –20°C until needed.

The rats were killed by decapitation at around 10:00 on the last day (day 28), after the urine sample had been collected. Serum was collected to measure biotin, and was stored at –20°C until needed. The liver, spleen, kidney, brain, and testis of each animal were removed, and a portion (about 0.5 g) was immediately treated as described next to measure biotin.

3.2.3 Analysis

Part of the 24 h urine samples was stored at –20°C. The content of biotin in the urine was directly measured by using *Lactobacillus plantarum* ATCC 8014[61]. To measure the free serum biotin content, 0.05 mL of serum was added to 1 mL of distilled water, and the mixture was heated for 5 min in a water bath at 100°C. After cooling to room temperature, the solution was centrifuged at 9000 *g* for 10 min at 4°C, and the resulting supernatant was used to measure biotin. To measure the total biotin content in the organs, a portion (about 0.5 g) of each organ (liver, spleen, kidney, brain,

and testis) was homogenized with two volumes of 2.25 M H_2SO_4 and then hydrolyzed by autoclaving for 1 h at 121°C and 2 atm. After cooling, the hydrolysate was centrifuged at 9000 *g* for 10 min at 4°C, and the resulting supernatant was used to measure biotin. To measure the free biotin content in the liver, a portion of the liver was homogenized with two volumes of a 0.05 M potassium phosphate buffer (pH 7.0), the homogenate was centrifuged at 9000 *g* for 10 min at 4°C, and the resulting supernatant was used to measure biotin.

3.2.4 Statistical analysis

Each value is expressed as the mean ± SEM. The statistical significance was determined by ANOVA, this being followed by Turkey's multiple-comparison test. $P<0.05$ was considered to be statistically significant. Graph Pad Prism4.0 (Graph Pad Software, San Diego, CA, USA) was used for all the analyses.

3.3 Results

The 0.00002% biotin diet was set as the control because the AIN-93 diet recommended by AIN contains 0.00002% biotin[11]. The food intake and body weight gain were not significantly different between the 0.04% biotin-added and control groups, whereas the food intake and body weight gain in the group with the >0.08% biotin-added diets were significantly lower than those in the control group (Fig. 3-1). Diarrhea was observed in the young rats fed with the >0.50% biotin-added diets.

One rat in four died with the 0.80% biotin-added diet, and two in four rats died with the 1.0% biotin-added diet. Therefore, the data for the 1.00% biotin-added diet group are not shown in Fig. 3-1.

Figure 3-2 shows the organ weight of the rats fed on the biotin diets. The brain weight was not significantly different among the seven groups. The weights of other organs, including the liver, kidney, spleen and testis, showed increasingly lower values in a dose-dependent manner. The organ weights in the 0.04% biotin-added group were the same as those in the control group, and all organ weights except the brain in the 0.50% and 0.80%-added groups were lower than those in the control group. The kidney weights in the groups with the >0.08% biotin-added diets were lower than those in the control group, the liver weights in the groups with the >0.10% biotin-added diets were lower, and the testis weights in the group with the >0.20% biotin-added diets were lower those in the control group.

The effect of excess biotin on the concentration of biotin in the urine is shown in Fig. 3-3A. The urinary excretion of biotin increased with increasing to dietary intake of biotin. The serum free biotin content also increased with increasing intake of biotin (Fig. 3-3B).

The concentrations of biotin in the brain, kidney, spleen and testis also increased with increasing intake of biotin (Fig. 3-4). The levels of biotin in the brain, kidney, and testis in the 0.04, 0.08, 0.10 and 0.20% biotin-added groups were significantly higher than those in the control groups. The spleen biotin content in the 0.04% biotin-added group was not significantly higher than that in

the controls, however, the biotin contents in the 0.08, 0.10 and 0.20% biotin adding groups were significantly higher than that in the control group.

3.4 Discussion

In the present study, an extremely high dose of biotin representing more than a 0.80% in the diet caused death, and more than 0.08% biotin-added diet retarded the growth of young rats. These results suggest that an excess biotin intake might cause some adverse effects on humans, and that setting UL for biotin would be important to prevent such dietary biotin-induced adverse effects. Although no adverse effects of biotin on humans have been reported, two studies have reported that subcutaneously administered biotin (50 and 100 mg/kg) to pregnant rats inhibited fetal and placental growth and resorption of fetuses and placentae[88,89]. The effects of excess biotin intake on the reproductive organs of male rats were not investigated in the present study, although the testis weights in the young rats fed with the diets containing more than 0.50% biotin were lower than those in the other groups. Whether an oral intake of high biotin by pregnant rats would affect the sex hormones, reproductive organs and fetal growth remains to be elucidated.

For increasing accumulation of biotin in the organs was observed as the amount of biotin administered was increased. This phenomenon might have been due to too great an amount of biotin than was possible to metabolize and excrete. It is suggested that this accumulation was associated with the retardation of growth. Hymes *et al*. have proposed a reaction mechanism by which the

enzyme, biotinidase (EC 3.5.1.12), mediates covalent binding of biotin to histones[58]. Biotinylation of histones might play a role in gene silencing[91], cell proliferation[56,92] and DNA repair or apoptosis[93]. Treatment of cell lines with a pharmacological concentration of biotin (10 pmoL/mL) for several weeks had only a moderate impact on biotinylation of histones, whereas the biotinylation of carboxylases was strongly correlated with the biotin concentration in the culture media[73,94-95]. A pharmacological dose of dietary biotin (100 mg/kg) has decreased the abundance of biotinylated carboxylase in rat liver[96]. It is unclear whether an excess biotin intake would affect the biotinylation of histones, and how these changes to histones and some carboxylases are related to the detrimental effect of an excess biotin intake.

The present experiment using young rats clearly indicated that an excessive oral intake of biotin retarded the body weight gain and food intake. Judging from the results of the body weight gain and food intake, the no observed adverse effect level (NOAEL) in young rats was 0.04% in the diet, and the lowest observed adverse effect level (LOAEL) was 0.08% in the diet. Young rats in the 0.04% biotin group consumed about 6.83 g/day of their diet during days 0 to 28, the mean body weight during that period being about 177.8g. Therefore, the biotin intake was calculated as 38.4 mg/kg body weight/day. Young rats in the 0.08% biotin group consumed about 11.76 g/day during days 0 to 28, the mean body weight during that period was being about 149.7 g. Therefore, the biotin intake was calculated as 79.2 mg/kg body weight/day. Although the present study clearly showed that the 79.2 mg/kg body weight/day oral intake of biotin caused adverse effects, the present

study investigated the acute, but not chronic, effects of excess biotin intake on the body weight gain, food intake, organ weight, organ biotin content, and not the histopathology nor production toxicity. Furthermore, the results of the present study were obtained from a limited number of animals, four rats in each group.

A single oral administration of 20 mg of biotin or 4.5 mg intravenously to healthy adults caused no adverse effect. An oral intake of 1.2 mg/day of biotin by healthy adults for 14 days also did not cause any adverse effect[97]. Since the data on adverse effects from a high biotin intake are not sufficient for a quantitative risk assessment, UL for biotin has not been derived in USA and Japan[44,98]. The data from human studies plausibly show the low risk of several mg of biotin intake, but our results clearly show that an excess intake of biotin increased the risk of adverse effects. A further study is therefore needed to collect enough data to set UL for biotin.

Table 3-1. Composition of the diets

	Control	0.04%	0.08%	0.1%	0.2%	0.5%	0.8%	1.0%
Casein	20	20	20	20	20	20	20	20
L-Methionine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
a-Cornstarch	46.9	46.86	46.82	46.8	46.7	46.5	46.3	46.2
Sucrose	23.4	23.4	23.4	23.4	23.4	23.3	23.2	23.1
Corn oil	5	5	5	5	5	5	5	5
Mineral mixture[a]	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mixture[a]	1	1	1	1	1	1	1	1
D-Biotin	0	0.04	0.08	0.1	0.2	0.5	0.8	1.0

Each value is expressed as g/100 g of diet.

[a] The compositions of the mineral and vitamin mixtures are described in ref. 90.

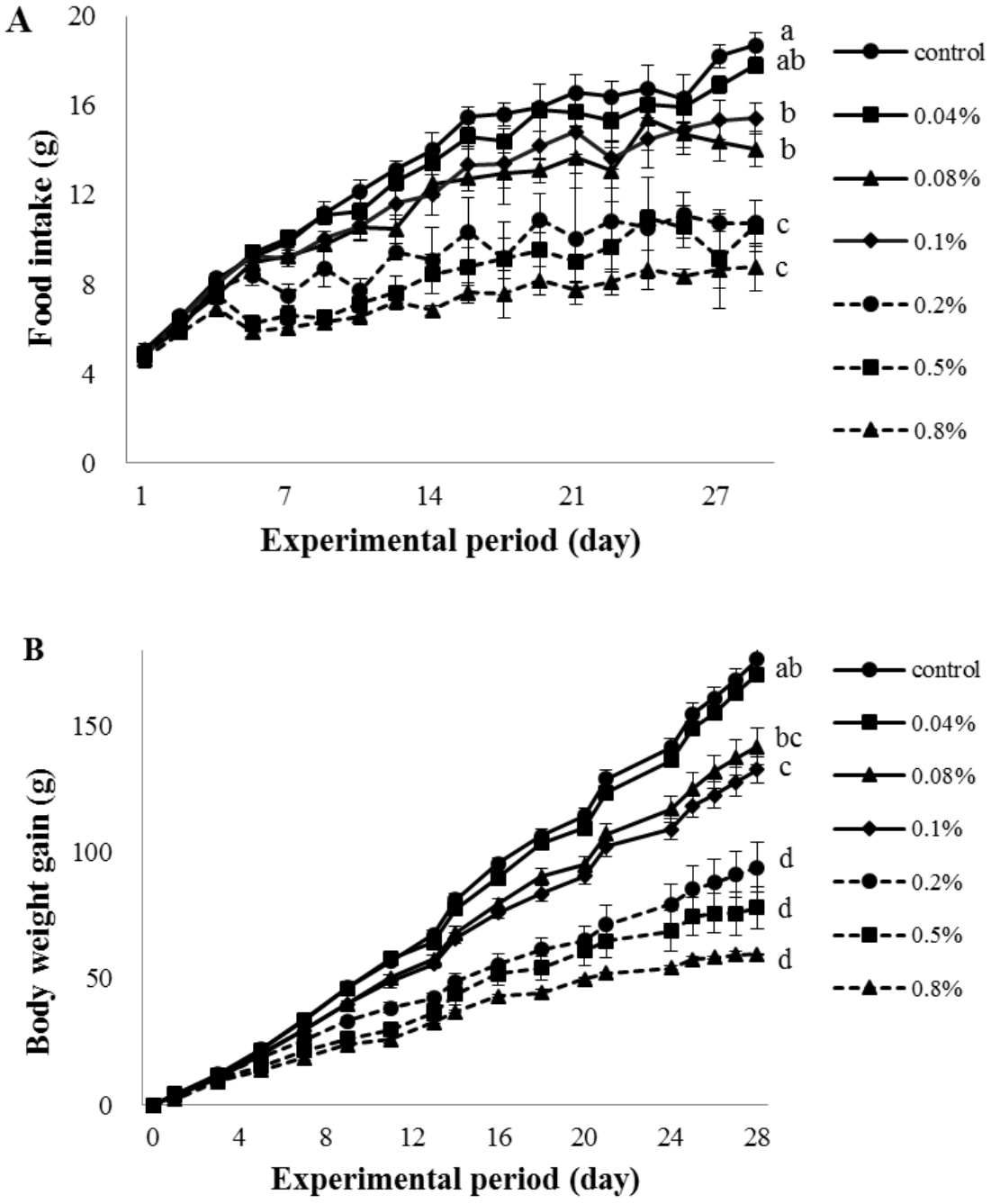

Fig. 3-1. Effects of biotin intake on the food intake (A) and body weight gain (B).

Each value is expressed as the mean ± SEM.

A different superscript letter means significant difference at $p < 0.05$.

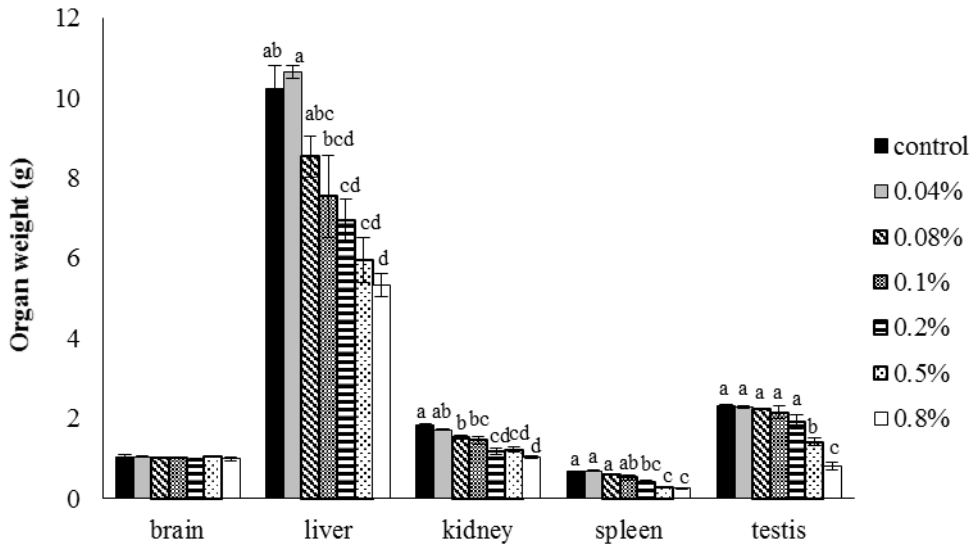

Fig. 3-2. Effects of biotin intake on the organ weights of rats.

Each value is expressed in g as the mean ± SEM.

A different superscript letter means significant difference at $p < 0.05$.

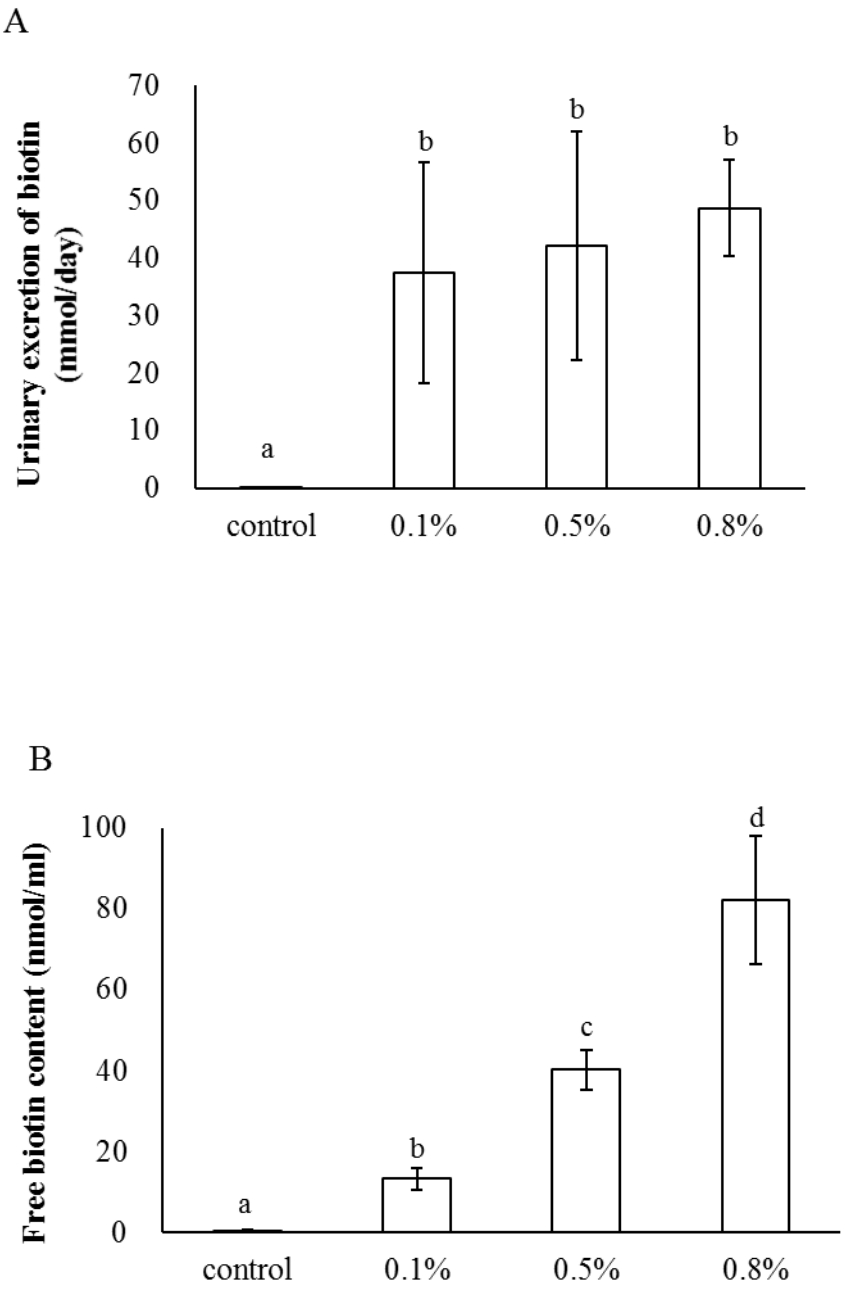

Fig. 3-3. Effects of Excessive Administration of Biotin on the Urinary Excretion of Biotin (A) and Free Biotin Content in the Serum (B).

The 24-hr urine samples were collected on the last day of the experiment, and then the serum was collected. Each bar is the mean ± SEM for 3 or 4 rats. A different superscript letter means significant difference at $p < 0.05$.

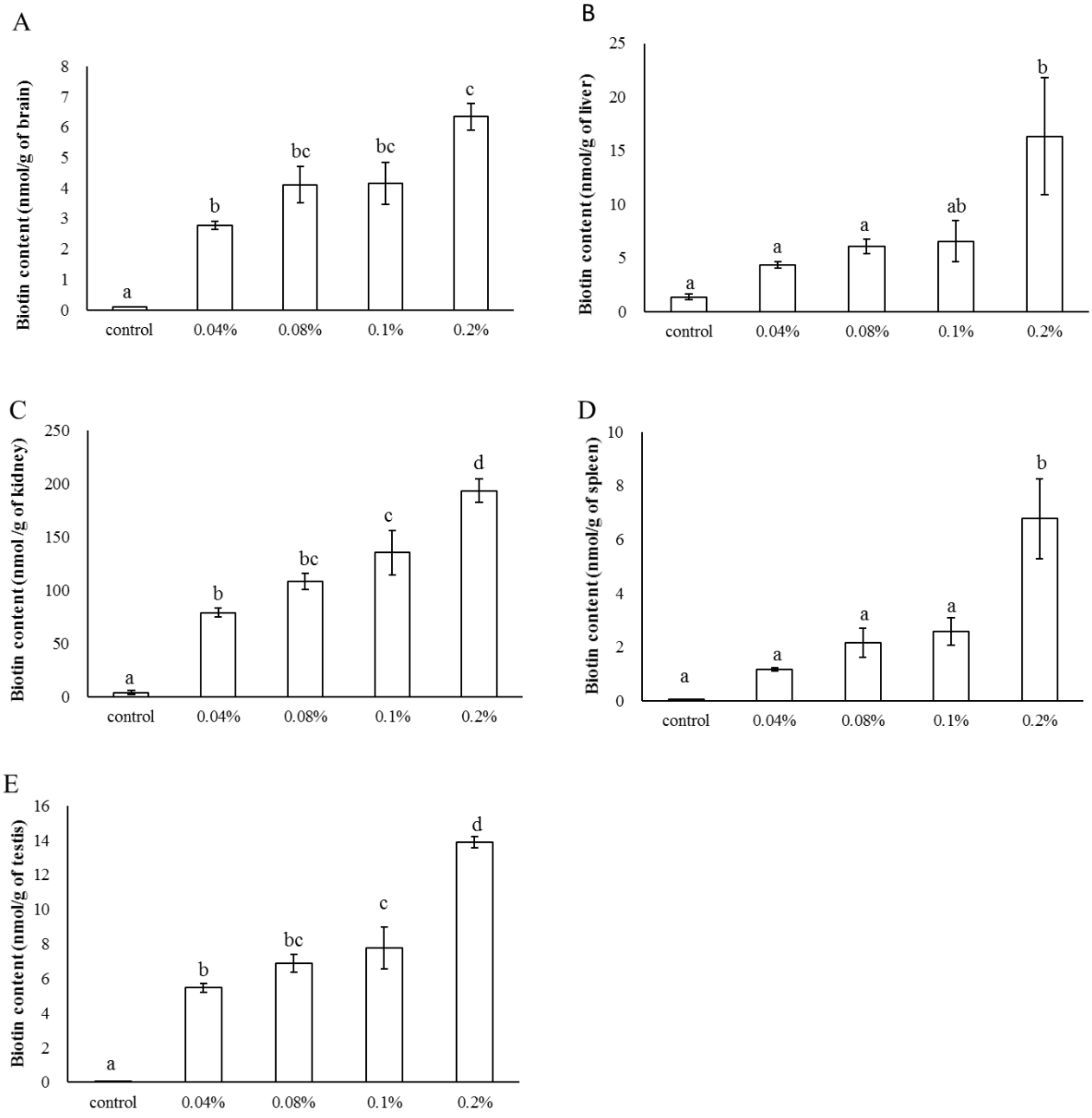

Fig. 3-4. Effects of Excessive Administration of Biotin on the Biotin Contents in the Brain (A), Liver (B), Kidney (C), Spleen (D), Testis (E).

The organs were collected the last day of the experiment. Each bar is the mean ± SEM for 3 or 4 rats. A different superscript letter means significant difference at $p < 0.05$.

Chapter 4: Excess Intake of Biotin Inhibits Spermatogenesis in Young Rats

4.1 Introduction

Biotin is known to play an important role in general metabolism and the normal development of mammals and birds. It is also crucial for maintaining reproductive functions and normal embryonic development[99]. However, to the best of our knowledge, few studies have examined the effects of excessive amounts of biotin during growth periods in mammals.

A repeated dose of biotin (25 mg/kg b.w. at morning and evening for one day or two consecutive days) administered subcutaneously to rats resulted in irregularities in the estrus cycle[88,100] as well as fetal and placental resorption in pregnant rats[100], and was accompanied by a decrease in uterine weight, reduced glycogen and protein levels in the uterus, and reduced protein levels in the liver. However, these studies cannot be regarded as conclusive for dietary biotin intake in humans due to the different route of administration.

Regarding the excess intake of biotin in humans, the administration of oral biotin at doses up to 100 mg/day to patients with holocarboxylase synthetase and biotinidase deficiencies did not result in adverse effects[45]. However, the 2010 version of the Dietary Reference Intakes for Japanese has presented no data on the tolerable upper intake level (UL) of biotin[44]. The risks associated with the excess intake of biotin have not yet been established in healthy humans, and any studies on the adverse effects of biotin have not been ethically permitted.

In rodents, the oral LD_{50} of biotin was reported to be >10 g/kg in mice[46], and >354 mg/kg in rats[47]; however, in these studies, the details were not provided. We previously reported that the oral administration of 1 g biotin/kg diet to pregnant mice during gestation had no effect on the rate of successful pregnancies[49]. Furthermore, we recently demonstrated that some rats fed a 0.8% (8 g biotin/kg diet) or higher biotin diet had diarrhea and died[101]. Regarding the effects of high-dose biotin on development and reproduction, Mitthelholzer[48] suggested that biotin at a dose totaling 50 mg/kg b.w. by a subcutaneous injection did not affect reproduction in female rats. Paul and Duttagupta[102,103] demonstrated that the acute administration of biotin (100 mg/kg b.w.) by subcutaneous injection during pregnancy caused the resorption of fetuses and placentae in rats. On the other hand, it remains unknown whether excessive amounts of biotin can affect reproduction in male animals. In the present study, we investigated the biochemical and histological effects of high-dose biotin (0.01% to 1.0%) intake on cell growth and sperm maturation during spermatogenesis in order to characterize a new function of biotin in reproduction in male rats.

4.2 Materials and methods

4.2.1 Animals and diets

Male Wistar rats (3 weeks old) were obtained from CLEA Japan Inc. (Tokyo, Japan). All animals were housed with CE-2 (CLEA Japan Inc.) for at least 1 week to acclimate them to the

animal room. Rats were then divided into five experimental groups: a control group (n=5), 0.01% biotin group (n=5), 0.1% biotin group (n=6), 1.0% biotin group (n=12), and pair-fed group (n=12). The control group was fed the CE-2 diet (containing 0.00004% biotin), and the experimental groups were fed the CE-2 diet supplemented with 0.01, 0.1, or 1.0% biotin. The intake of biotin in the 1.0% biotin-added rat corresponds to a dose of 990 mg/kg b.w./day. Pair-fed rats were fed a control diet that was equal in calories to the amount ingested by the 1.0% biotin group, as daily food intake was significantly decreased in the 1.0% biotin group. The diet amount to be given to the pair-fed rats was calculated as the mean food intake of all rats in the 1.0% biotin group during the previous week. Male rats were given these diets and distilled water *ad libitum* for 6 or 8 weeks. Half of the rats in the 1.0% biotin and pair-fed groups were fed for 8 weeks, while the others were fed for 6 weeks. The temperature in the animal room was maintained at approximately 22°C with 60% humidity and a 12 h light/dark cycle (lights on between 9:00-21:00). Body weights and food intakes were measured every week. Urine samples and feces over 24 h periods were collected every other week and stored at -40°C until analyzed. The rats were anesthetized and killed after 6 weeks or 8 weeks of feeding (on day 42 or day 56, respectively). Blood was collected, centrifuged for 15 min at 800 × *g*, and serum was stored at -40°C before use. The liver, kidneys, brain, testis, and spleen were sampled and weighed.

All experimental procedures including the care and treatment of rats described in this study were approved by the Institutional Animal Care and Use Committee of the School of Human Science and Environment, University of Hyogo.

4.2.2 Sampling

Samples of feces were dried at 70°C in a forced-air oven, weighed, and crushed into powder. 1 g of each sample was added to 9 mL of distilled water, mixed well, centrifuged for 10 min at 800 × *g*, and the supernatant was then collected. To measure biotin contents in the organs, the liver and testes were homogenized with a sodium phosphate buffer (pH = 7.4). After centrifuging at 3,000 rpm, the supernatant was collected.

4.2.3 Biochemical analysis

Biotin contents in the serum, organs, urine, and feces were determined using a microtiter plate by the microbiological assay with *Lactobacillus plantarum* ATCC 8014. *L. plantarum* was cultured with samples for 20 h and determined at 610 nm. As biotin in organs is partially contained in a protein-binding form, total and free biotin were separately determined. To determine total biotin, 100 µL of the sample solution was hydrolyzed with 2.25 M H_2S0_4 for 121°C (2 atoms) for 60 min and neutralized with 4.5 M NaOH. Free biotin was determined without hydrolysis. Biotin concentrations were expressed as nmol/g of the protein. Total protein contents in the organ samples

were assayed by the Bradford method using a commercial kit (Bio-rad Laboratories, California, USA).

To measure testosterone levels, the testes were homogenized with six volumes of 0.01 M phosphate buffered saline, and testosterone was then extracted from 1 mL of the homogenate with a diethyl ether. Samples were then reconstituted in 1 mL of an enzyme immunoassay (EIA) buffer and testosterone levels were determined using a Testosterone EIA kit (Cayman Chemical Company, Michigan, USA).

4.2.4 Histological and morphological analyses

The testes were fixed in a 10% neutralized formalin solution, cut into 5 µm sections, placed on glass slides, and stained with hematoxylin-eosin for the histological examination of early spermatogenesis. The diameter of the seminiferous tubules was also measured under a microscope at 80× magnification. To detect apoptotic cells *in situ* at the single cell level, testicular sections were treated using the TUNEL method (Apop-Tag Peroxidase In Situ Apoptosis Detection Kit, Chemicon International, Massachusetts, USA).

The left cauda epididymis was gently minced in a physiological saline solution at 37°C in order to prepare a sperm suspension for determining the sperm count and sperm morphology. Sperm preparations for the sperm morphology were smeared on a slide glass and air dried it, before it was fixed in Carnoy’s solution for over an hour, and then stained for 30 min in 1% eosin Y. Sperm

preparations for the sperm count were fixed with 10% formalin. The sperm count was assessed using a Bürker-Türk haemocytometer. Abnormalities in the shape of sperm were classified according to the criteria described by Mori[104] and Wyrobek et al.[105] with slight modifications[106]. The analysis of sperm morphology was previously evaluated as a useful method for screening environmental mutagens.

4.2.5 Statistical analysis

Each value was expressed as the mean ± SD. The statistical significance was determined by two-way ANOVA, this being followed by the Turkey's multiple-comparison test. $P < 0.05$ was considered to be statistically significant. All the analyses were performed on a personal computer using a standard statistical package (Statcel Ver. 3, Tokyo, Japan).

4.3 Results

4.3.1 Body weight and dietary intake

No significant differences were observed in food intake or body weight between the 0.01%, 0.1% biotin, and control groups (Fig. 4-1). However, growth was less in the 1.0% biotin group than in the control group. Hair loss and mucosal erosion were observed in the rats of this group after 4 weeks of feeding, and only one died over a period of 6 weeks.

Figure 4-2 shows the organ weights of rats fed the high-dose biotin diets. The liver and spleen weights were significantly lower in the 0.01% biotin group than in the control group. The kidney, brain and testis weights were significantly lower in the 1.0% biotin group than in the pair-fed group. There is no statistically significant difference between the control and 0.1% biotin group.

4.3.2 Biochemical findings

The biotin contents in the testis and liver are shown in Fig. 4-3. Regarding the biotin content in the liver, both total and free biotin contents increased with biotin intake. However, the content of the protein-binding form of biotin in the liver did not change. In the liver, over 96.0% of total biotin in the control group, and 69.0, 38.0, 24.6, and 99.3% in the 0.01, 0.1, 1.0% biotin, and pair-fed groups, respectively, existed in the protein-binding form. On the other hand, biotin contents in the testes also increased with an increase in the intake of biotin. Over 90% of total biotin in the testes in all the biotin groups existed as the free form of biotin.

4.3.3 Morphological and histological findings

The number of sperm in the caudal epididymis in rats was examined. An examination of sperm revealed that the sperm count was $34 \pm 25 \times 10^6$/mL in the pair-fed group, while that in the 1.0% biotin group was less than 1 sperm/mL. The morphological analysis of sperm revealed a significant

increase in the number of sperm with an abnormal head, mainly round heads, in the 1.0% biotin rat (Fig. 4-4).

The histological analysis showed that only a few spermatogonia and no spermatocytes existed in the 1.0% biotin group (Fig. 4-5). The diameter of the seminiferous tubules was 230.0 ± 24.5 μm in the 1.0% biotin group, which was significantly lower than that in the pair-fed group (342.5 ± 26.3 μm) (Fig. 4-6). These results indicated that the diameter of the seminiferous tubules in the spermatogenic epithelium of the 1.0% biotin group was affected by the administration of a high dose of biotin. Apoptosis was not observed in the testes of the pair-fed and 1.0% biotin groups. No significant differences were observed in testosterone levels in the testes among the groups (Fig. 4-7).

4.4 Discussion

A number of studies reported that some factors, such as nutrients[107], stress[108], and temperature[109], are involved in the regulation of spermatogenesis. Vitamins have an important role in the production of spermatozoa. For example, a deficiency in the water-soluble vitamin, vitamin B_{12}, was shown to induce histological changes in the testes of rats, resulting in aplasia in sperm[110-113]. A vitamin C deficiency also disturbs spermatogenesis; therefore, the dietary prescription of vitamin C has been shown to protect germ cells from oxidative stress throughout spermatogenesis in humans[7]. Fat-soluble vitamins have also been associated with the endocrinological function and

development of the testes. A previous study demonstrated that a vitamin D deficiency significantly reduced the mating ratio in rats, resulting in a low reproductive capacity[1]. A vitamin A deficiency in rats has also been shown to lead to low serum testosterone levels and suppresses the ability of spermatogonia to differentiate[4,114-115]. On the other hand, excess vitamin A levels in rats reduced the weight of the testes and enlarged the nuclei of spermatocytes[5]. These findings indicated that although vitamins are essential for spermatogenesis, the mechanisms underlying the roles of vitamins in male reproduction remain unclear.

The importance of biotin in reproduction and prenatal development has been investigated in experimental animals. We previously demonstrated that a biotin deficiency during gestation in mice caused a markedly high incidence of congenital malformations such as cleft palate, micromelia, and micrognathia in fetuses[37-39,66-67]. On the other hand, the acute dose of biotin (25 mg/kg b.w. at morning and evening for one day or two consecutive days by subcutaneous injection) to rats during pregnancy inhibited fetal and placental growth and increased the resorption of fetuses and placentae[88,100]. Báez-Saldaña et al.[116] also reported that the oral administration of biotin (16.6 mg/kg b.w./day for 8-9 weeks) decreased the number of primary and Graafian follicles in mice. We previously reported that a 1.0% biotin diet affected the growth of young mice[49]. Based on the findings of these studies, we speculated that high doses of biotin may affect reproduction in males. In the present study, we concluded that an extremely high-dose of biotin, representing a 1.0% biotin diet, caused death and affected the testicular system. The testis weights in rats fed the 1.0% biotin

diet were lower than those in the other groups. Furthermore, the 1.0% biotin diet increased the incidence of sperm with morphologically abnormal shapes and inhibited spermatogenesis in rats.

Spermatogenesis is primarily dependent on testosterone, which has been linked to cell division and cell growth in spermatogenesis. Paulose et al.[117] previously demonstrated that testicular and serum levels of testosterone were decreased in biotin-deficient rats. Thus, in addition to testosterone and follicle-stimulating hormones (FSH) for normal interactions in Leydig cells, Sertoli cells, and peritubular cells, biotin may be required in testicular development such as meiosis and sperm maturation. In the present study, we observed that the testicular level of testosterone was not significantly decreased in the 1.0% biotin rats. Veeramachaneni et al.[118] suggested that testosterone may not affect spermatogenesis directly. Song et al.[119] demonstrated that the modification pattern of histone H3 was subjected to dynamic changes and specific to a certain stage of germ cell differentiation during mouse spermatogenesis. Epigenetic effects on testicular function and spermatogenesis represents a new study field. Recently, a novel posttranslational modification has been identified: covalent binding of biotin to lysine residues in histones[30]. Biotinylation of histones plays a role in cell proliferation, gene silencing and cellular response to DNA damage. Low levels of histone biotinylation have been linked to increased frequency of retrotransposition events, suggesting a role for histone biotinylation in chromosomal stability[120]. Thus, these findings indicated that changes in histone biotinylation levels in high-dose biotin added rat may affect spermatogenesis. The biotin content was increased in the organs in the present study as the amount

of biotin administrated exponentially increased. Therefore, the accumulation of free biotin in the testes may be associated with the inhibition of reproduction in males. Further studies are needed to clarify the mechanism by which biotin interferes with spermatogenesis.

No sperm was also detected in the caudal epididymis in rats in our other study, and multinucleated giant cell and apoptosis-like cell death in the spermatogenic epithelium of the seminiferous tubules was histologically detected even in the 0.5% biotin group (unpublished data). Thus, spermatogenesis may be inhibited at a dose level lower than 1.0% as well. We intend to examine doses between 0.1 and 1.0% and incorporate the measurement of sperm production and storage with sperm quality and other biochemical parameters in order to clarify the threshold level of biotin for spermatogenic inhibition. Furthermore, a recovery test is needed to determine whether biotin-induced effects are reversible.

In conclusion, this is the first study to show that dietary high-dose biotin intake affected reproduction in males and inhibited the maturation of spermatogenic cells. We selected a high-dose of biotin because the purpose of this study was to characterize a new function of biotin in reproduction in males. However, the results of the present study were obtained from a limited number of rats. Therefore, further studies are needed using a larger number of animals and other species. A high dietary dose of biotin may lead to some adverse effects in humans; however, the dose used in the present study was too large to determine the No observed adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL), and setting UL for biotin may be

important for preventing such dietary biotin-induced adverse effects. Therefore, more definitive experiments are required before setting UL for biotin.

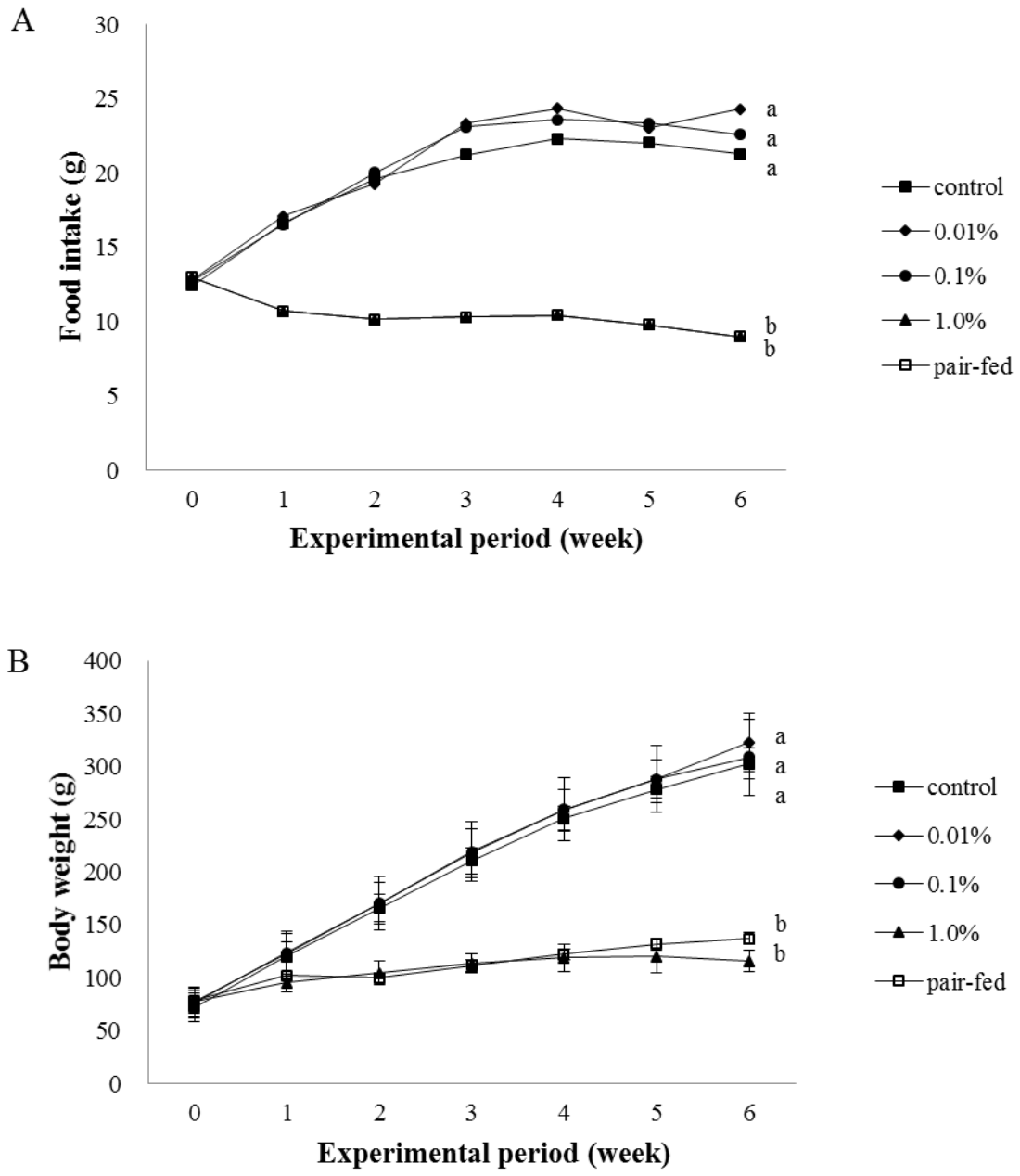

Fig. 4-1 Effects of dietary high-dose biotin on food intake (A) and body weight (B).

Each value is expressed as the mean ± SD (n=5-6). A different superscript letter means significant difference at $p < 0.05$.

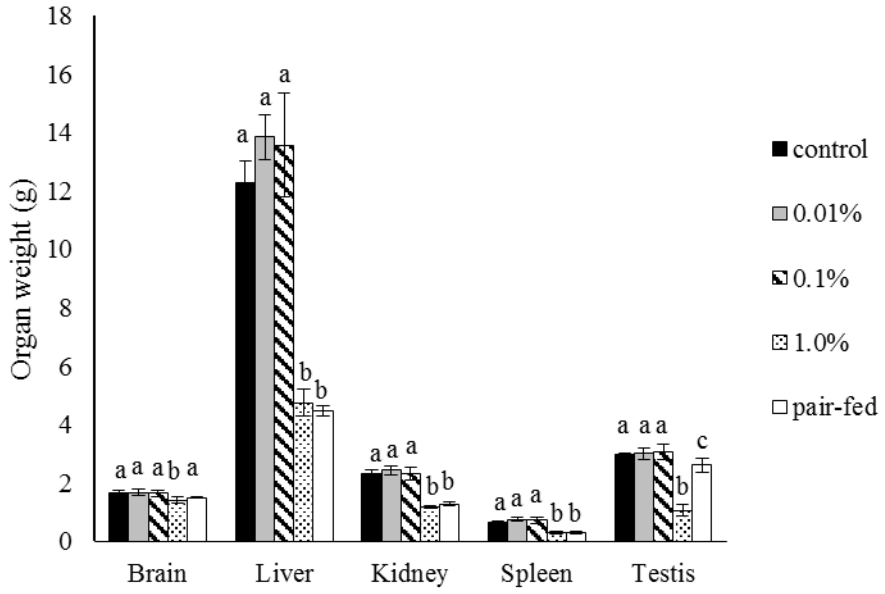

Fig. 4-2 Effects of dietary high-dose biotin on organ weights in rats.

Each bar represents the mean ± SD. A different superscript letter means significant difference at $p < 0.05$.

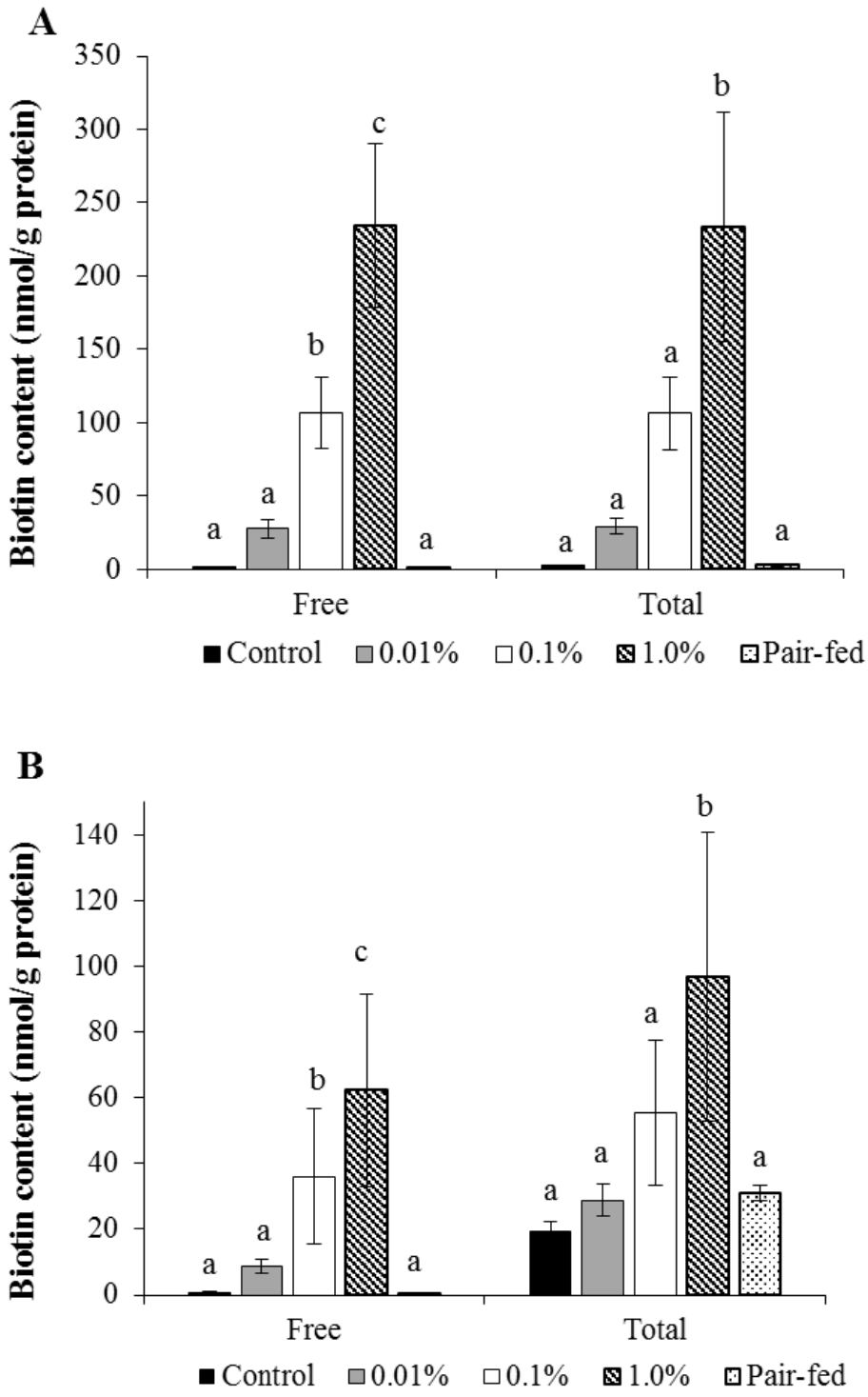

Fig. 4-3 Effects of dietary high-dose biotin on biotin contents in the testis (A) and liver (B). Each bar represents the mean ± SD. A different superscript letter means significant difference at $p < 0.05$.

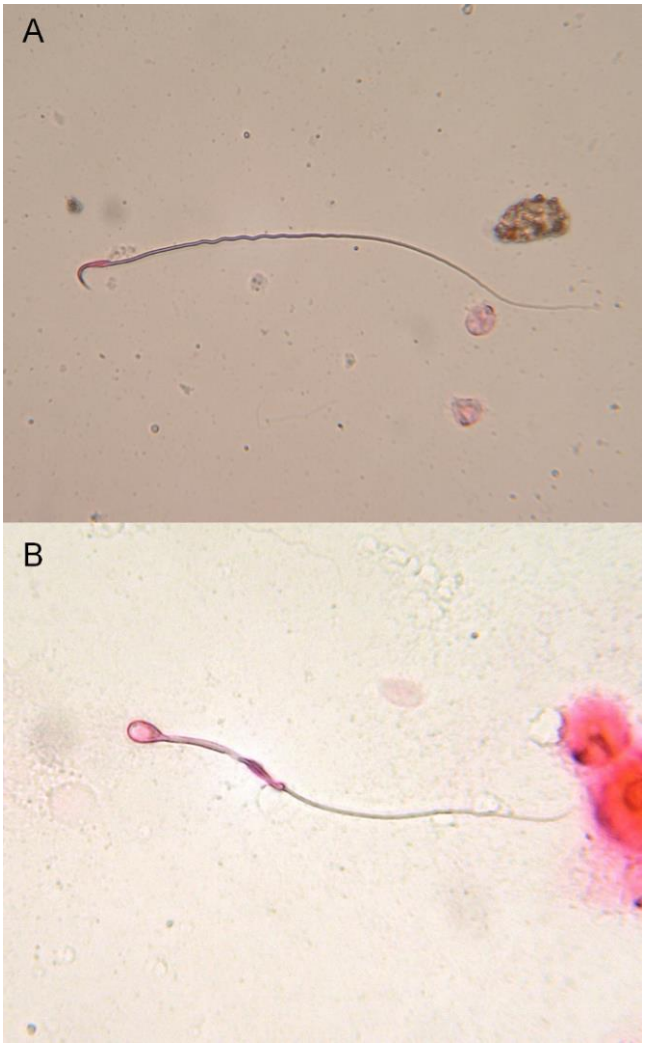

Fig. 4-4 Effects of dietary high-dose biotin on sperm morphology.

Sperm preparations were stained in 1% eosin Y. An analysis of the sperm morphology was evaluated as a useful method for screening environmental mutagens. (A) Sperm heads in the pair-fed group. (B) Sperm heads in 1.0% biotin group were abnormally rounded.

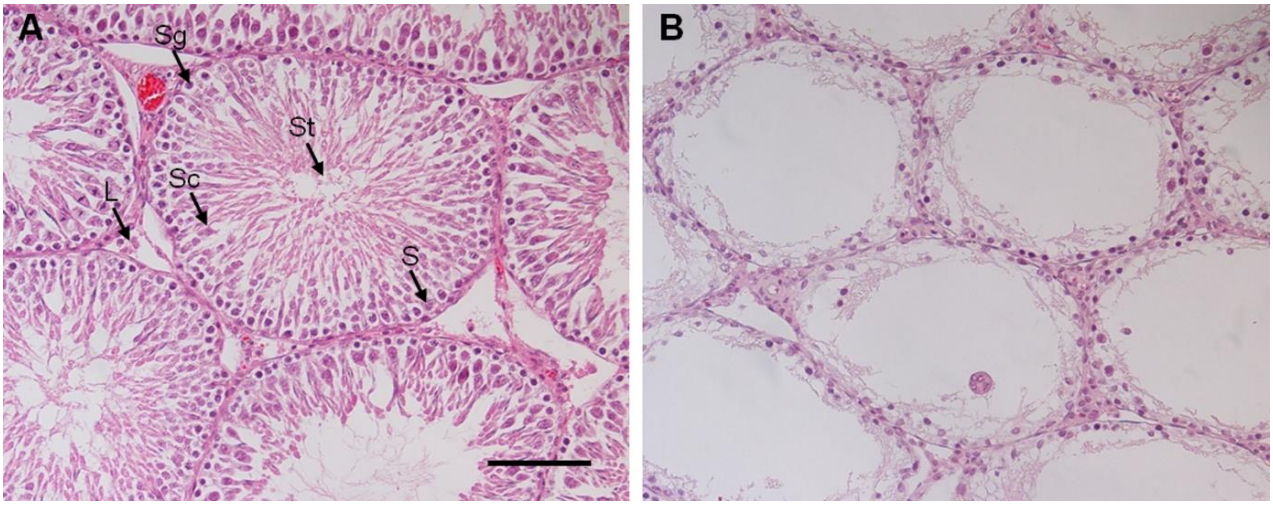

Fig. 4-5 Effects of dietary high-dose biotin on the testes in rats.

The testes were collected on the last day of the experiment. Testes were stained with hematoxylin and eosin in the pair-fed group (A) and 1.0% biotin group (B). S, Sertoli cells; Sg, spermatogonia; Sc, spermatocyte; St, spermatid and L, Leydig cells. Scale bar = 100 µm.

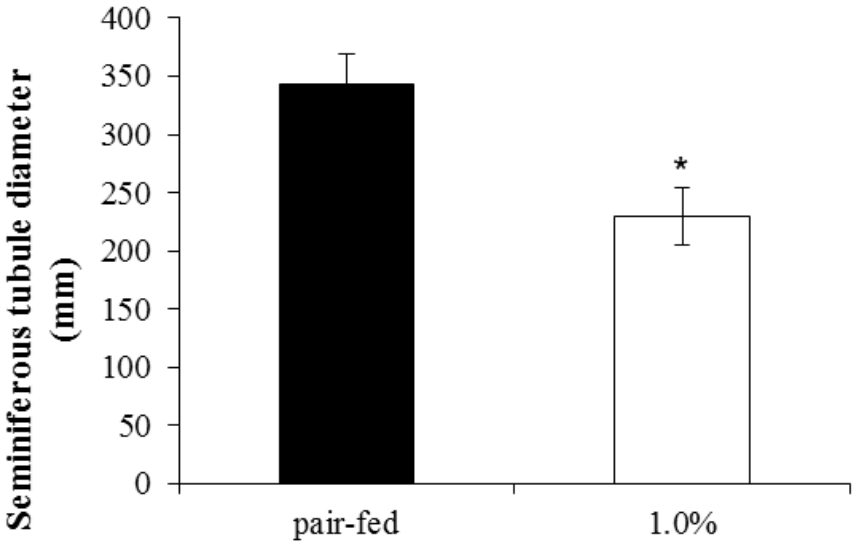

Fig. 4-6 Effects of dietary high-dose biotin on seminiferous tubule diameter in rats.

Each bar represents the mean ± SD. *P < 0.001, compared with the pair-fed group.

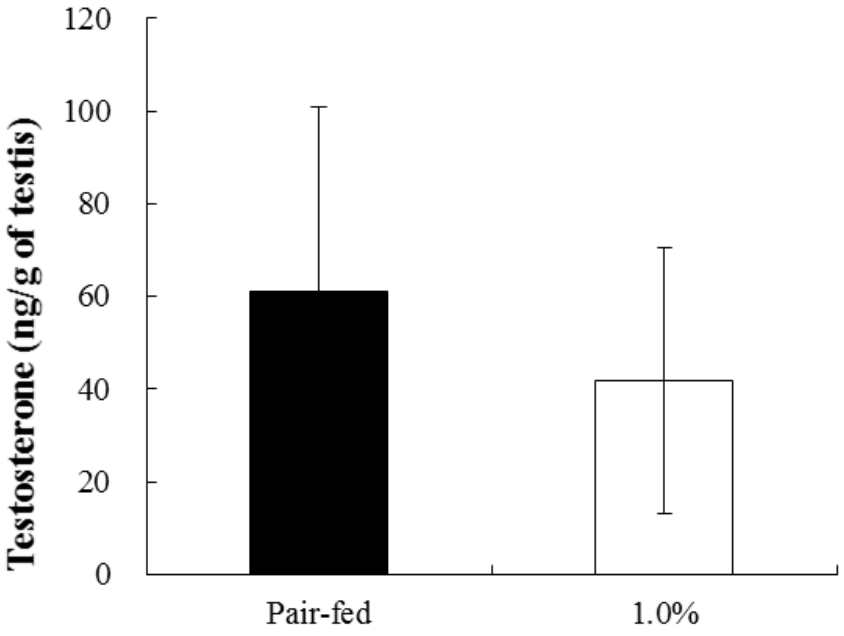

Fig. 4-7 Effects of dietary high-dose biotin on the testicular testosterone level in rats.

Each bar represents the mean ± SD.

Chapter 5. Conclusion

To clarify the effects of low and excess biotin intake on female and male reproduction in rodents, three studies were performed. The first study examined whether low intake of biotin during pregnancy affects the expression of any gene that plays an important role in maintaining biotin homeostasis in mice (Chapter 2), the second study examined whether the excess intake of biotin (0.01% to 1.0%) affects early growth in young male rats (Chapter 3) and the third study investigated the biochemical and histological effects of the excess intake of biotin (0.01% to 1.0%) on cell growth and sperm maturation during spermatogenesis in male rats (Chapter 4).

Numerous studies have shown that biotin is essential for reproduction and fetal development in mammals[37-39]. However, the relationship between biotin and fetal development is not well known. As described in chapter 2, the first study demonstrated that the expression of SMVT was significantly increased at mRNA and protein levels in the placenta of the biotin-deficient group. We confirmed that low intake of biotin during pregnancy changes SMVT gene and protein expression. These results demonstrate that maternal biotin deficiency may be associated with biotin transporter upregulation accompanied by the abnormal development of fetuses.

The risks associated with the excess intake of biotin have not yet been established in healthy humans. As described in chapter 3, a second study demonstrated that excess intake of biotin (more than 0.08% biotin/kg diet) retarded the growth of young male rats. Additionally, testis weights in the rats fed diets containing more than 0.5% biotin were lower than those in the control. These

results indicate that the excess intake of biotin may affect testicular function.

On the basis of the results of the second study, we focused on the effects of the excess intake of biotin on male reproduction. The importance of biotin in reproduction and fetal development has been investigated in experimental animals[37-40,66-67]. However, it remains unknown whether excessive amounts of it can affect reproduction in male animals. As described in chapter 4, the third study showed that the excess intake of biotin (1.0%) caused reductions in the numbers of mature sperm and spermatogonia. We thus confirmed that the excess intake of biotin inhibits spermatogenesis. Additionally, epigenetic effects on testicular function and spermatogenesis have recently been reported[119]. These results indicate that biotin may play a role in the epigenetic effects in male reproduction, mediating the biotinylation of histone.

In conclusion, we demonstrated that a low level of maternal biotin intake changes the expression of SMVT in the placenta and the excess intake of biotin inhibits spermatogenesis. These results indicate that biotin may play a role in the epigenetic effects in female and male reproduction, mediating the biotinylation of histone. We confirmed that biotin is essential for reproduction and development in both males and females, but its excess intake may adversely affect the reproductive system. Further study is needed to determine the recommended intake of biotin for pregnant women and to set a tolerable upper intake level for it.

References

1. Kwiecinski GG, Petrie GI and DeLuca HF: Vitamin D is necessary for reproductive functions of the male rat. J Nutr, 119: 741-744, 1989.
2. Wang S, Wang G, Barton BE, Murphy TF and Huang HF: Beneficial effects of vitamin E in sperm functions in the rat after spinal cord injury. J Andro, 28: 334-341, 2007.
3. Wolbach SB and Howe PR: Tissue changes following deprivation of fat-soluble A vitamin. J Exp Med, 42: 753–777, 1925.
4. Hogarth CA and Griswold MD: The key role of vitamin A in spermatogenesis. J Clin Invest, 120: 956–962, 2010.
5. Misro MM, Jena S and Paul PK: Effect of vitamin A excess on germ cell development in prepubertal rat testis. Indian J Exp Biol, 35: 576-580, 1997.
6. Begum H, Moniruddin ABM and Nahar K: Environmental and nutritional aspect in male infertility. J Med, 10: 16-19, 2009.
7. Angulo C, Maldonado R, Pulgar E, Mancilla H, Cordova A, Villarroel F, Castro MA and Concha ll: Vitamin C and oxidative stress in the seminiferous epithelium. Biol Res, 44:169-180, 2011.
8. Wallock LM, Tamura T, Mayr CA, Johnston KE, Ames BN and Jacob RA: Low seminal plasma folate concentrations are associated with low sperm density and count in male smokers and nonsmokers. Fertil Steril, 75: 252–259, 2001.
9. Boxmeer JC, Smit M, Utomo E, Romijn JC, Eijkemans MJ, Lindemans J, Laven JS, Macklon NS, Steegers EA and Steegers-Theunissen RP: Low folate in seminal plasma is associated with increased sperm DNA damage. Fertil Steril, 92: 548–556, 2009.

10. Yamamoto T, Jaroenporn S, Pan L, Azumano I, Onda M, Nakamura K, Watanabe G and Taya K: Effects of pantothenic acid on testicular function in male rats. J Vet Med Sci, 71: 1427–1432, 2009.

11. Mori K, Kaido M, Fujishiro K, Inoue N and Koide O: Effects of megadoses of pyridoxine on spermatogenesis and male reproductive organs in rats. Arch Toxicol, 66: 198-203, 1992.

12. Maxwell JD, Ang L, Brooke OG and Brown IRF: Vitamin D supplements enhance weight gain and nutrional status in pregnant Asians. Br J Obstet Gyneaecol, 88: 987–991, 1981.

13. Marya RK, Rathee S, Lata V and Mudgil S: Effects of vitamin D supplementation in pregnancy. Gynecol Obstet Invest, 12: 155–161, 1981.

14. Brunvand L, Quigstad E, Urdal P and Haug: Vitamin D deficiency and fetal growth. Early Hum Dev, 45: 27–33, 1996.

15. Scholl TO, Chen X, Sims M and Stein TP: Vitamin E: maternal concentrations are associated with fetal growth. Am J Clin Nutr, 84:1442-1448, 2006.

16. Clagett-Dame M and Knutson D: Vitamin A in reproduction and development. Nutrients, 3: 385-428, 2011.

17. Hogeveen M, Blom HJ and den Heijer M: Maternal homocysteine and small-for-gestational-age offspring: systematic review and meta-analysis. Am J Clin Nutr, 95: 130-136, 2012.

18. Committee on Nutritional Status during Pregnancy and Lactation, Institute of Medicine. Nutrition during pregnancy. Washington DC, National Academy Press, 1990.

19. Morita H, Omori Y and Oshima Y: Biological Studies on Pantethine: VIII. Influence of drugs on estrous cycle and reproductive performance in pantothenic acid deficient mice. Jpn J Vet

Sci 32: 201–207, 1970.

20. Roecklein B, Lewin SW, Comly M and Mukherjee AB: Intrauterine growth retardation induced by thiamine deficiency and pyrithiamine during pregnancy in the rat. Am J Obstet Gynecol, 151: 455-460, 1985.
21. Kalter H and Warkany J: Congenital malformations in inbred strains of mice induced by riboflavin-deficient, galactoflavin-containing diets. J Exp Zool, 136: 531–565, 1957.
22. Reeves PG, Nielsen FH and Fahey GC Jr: AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr, 123: 1939–1951, 1993.
23. Alton-Mackey MG and Walker BL: Graded levels of pyridoxine in the rat diet during gestation and the physical and neuromotor development of offspring. Am J Clin Nutr, 246: 420-428, 1973.
24. Boas MA: The effect of desiccation upon the nutritive properties of egg-white. Biochem J, 21: 712-724, 1927.
25. Knowles JR: The mechanism of biotin-dependent enzymes. Ann Rev Biochem, 58: 195-221, 1989.
26. Kim KH, McCormick DB, Bier DM and Goodridge AG: Regulation of mammalian acetyl-coenzyme A carboxylase. Ann Rev Nutr, 17: 77-99, 1997.
27. Wood HG and Barden RE: Biotin enzymes. Ann Rev Biochem, 46: 385-413, 1977.
28. Edited by Zempleni J, Wijeratne SSK, Kuroishi T: Biotin, Present Knowledge in Nutrition, 10th Edition. 359-374, International Life Sciences Institute, Washington DC, 2012.
29. Zempleni J: Uptake, localization, and noncarboxylase roles of biotin. Annu Rev Nutr, 25: 175-

196, 2005.

30. Kothapalli N, Camporeale G, Kueh A, Chew YC, Oommen AM, Griffin JB, Zempleni J: Biological functions of biotinylated histones. J Nutr Biochem, 16: 446-448, 2005.
31. McMahon R: Biotin in metabolism and molecular biology. Annu Rev Nutr, 22: 221–239, 2002.
32. Zempleni J, Wijeratne SSK and Hassan YI: Biotin. Biofactors, 35: 36–46, 2009.
33. Said HM, Thuy LP, Sweetman L, Schatzman B: Transport of the biotin dietary derivative biocytin (N-biotinyl-L-lysine) in rat small intestine. Gastroenterology, 104: 75–80, 1993.
34. Wolf B, Grier RE, Allen RJ, Goodman SI and Kien SL: Biotinidase deficiency: the enzymatic defect in late-onset multiple carboxylase deficiency. Clin Chim Acta, 131: 273–281, 1983.
35. Prasad PD, Wang H and Huang W et al.: Molecular and functional characterization of the intestinal Na^+-dependent multivitamin transporter. Arch Biochem Biophys, 366: 95-106, 1999.
36. Wang H, Huang W, Fei YJ et al.: Human placental Na+-dependent multivitamin transporter. Cloning, functional expression, gene structure, and chromosomal localization. J Biol Chem, 274: 14875-14883, 1999.
37. Watanabe T: Teratogenic effects of biotin deficiency in mice. Teratology, 113: 574-581, 1983.
38. Watanabe T and Endo A: Teratogenic effects of avidin-induced biotin deficiency in mice. Teratology, 30: 91-94, 1984.
39. Watanabe T and Endo A: Species and strain differences in teratogenic effects of biotin deficiency in rodents. J Nutr, 119: 255-261, 1989.
40. Watanabe T: Dietary biotin deficiency affects reproductive function and prenatal development in hamsters. J Nutr, 123: 2101-2108, 1993.
41. Mock NI, Malik MI, Stumbo PJ, Bishop WP and Mock DM: Increased urinary excretion of 3-

hydroxyisovarelic acid and decreased urinary excretion of biotin are sensitive early indicators of decreased biotin status in experimental biotin deficiency. Am J Clin Nutr, 65: 951-958, 1997.

42. Mock DM, Stadler DD, Stratton SL and Mock NI: Biotin status assessed longitudinally in pregnant women. J Nutr, 127: 710-716, 1997.

43. Zempleni J, Hassan YI and Wijeratne SS: Biotin and biotinidase deficiency. Expert Rev Endocrinol Metab, 3: 715-724, 2008.

44. Ministry of Health, Labour and Welfare. 2009. Dietary Reference Intakes for Japanese, 2010 version.

45. Baumgartner ER and Suormala T: Multiple carboxylase deficiency: inherited and acquired disorders of biotin metabolism. Internat J Vitam Nutr Res, 67: 377-384, 1997.

46. Informatics Inc. Scientific literature reviews on generally recognized as safe (GRAS) food ingredients. Biotin. Prepared for the FDA. Dated June. NTIS PB-234 890, 1974.

47. Bonjour JP. Biotin. In Handbook of vitamins. 2nd edition. New York: Marcel Dekker. p. 393-427, 1991.

48. Mittelholzer E: Absence of influence of high doses of biotin on reproductive performance in female rats. Internat J Vit Nutr Res 46:33-39, 1976.

49. Watanabe T: Morphological and biochemical effects of excessive amounts of biotin on embryonic development in mice. Experientia 52:149-154, 1996.

50. Mock DM, Mock NI, Lombard KA and Nelson RP: Disturbances in biotin metabolism in children undergoing long-term anticonvulsant therapy. J Pediatr Gastroenterol Nutr, 26: 245–250, 1998.

51. Watanabe T, Masaki T, Yuasa M, Morimoto M and Sawamura H: Estimate of the dietary intake of biotin in infants prescribed special therapeutic infant formulas in Japan. Int J Anal Bio-Sci, 1: 60-70, 2013.

52. Watanabe T, Oguchi K, Ebara S and Fukui T: Measurement of 3-hydroxyisovarelic acid in urine of biotin-deficient infants and mice by HPLC. J Nutr, 135: 615-618, 2005.

53. Watanabe T, Nagai Y, Taniguchi A, Ebara S, Kimura S and Fukui T: Effects of biotin deficiency on embryonic development in mice. Nutrition, 25: 78-84, 2009.

54. Ghosal A, Lambrecht N, Subramanya SB, Kapadia R and Said HM: Conditional knockout of the *SLC5a6* gene in mouse intestine impairs biotin absorption. Am J physiol Gastrointest Liver Physiol, 304: 64-71, 2013.

55. Leon-Del-Rio A and Gravel RA: Sequence requirements for the biotinylation of carboxyl-terminal fragments of human propionyl-CoA carboxylase alpha subunit expressed in Escherichia coli. J Biol Chem, 269: 22964-22968, 1994.

56. Narang MA, Dumas R, Ayer LM and Gravel RA: Reduced histone biotinylation in multiple carboxylase deficiency patients: a nuclear role for holocarboxylase synthetase. Hum Mol Genet, 13: 15-23, 2004.

57. Chauhan J and Dakshinamurti K: Role of human serum biotindiase as biotin-binding protein. Biochem J, 256: 265-270, 1998.

58. Hymes J, Fleischhauer K and Wolf B: Biotinylation of histones by human serum biotinidase: assessment of biotinyl-transferase activity in sera from normal individuals and children with biotinidase deficiency. Biochem Mol Med, 56: 76–83, 1995.

59. Wolf B: The Metabolic and Molecular Bases of Inherited Disease, 3935-3962, McGraw-Hill

Medical Publishing Division, New York, (2001)

60. Watanabe T and Endo A: Teratogenic effects of maternal biotin deficiency in mouse embryos examined at midgestation. Teratology, 42: 295-300, 1990.

61. Fukui T, Iinuma K and Oizumi J: Agar plate method using *Lactobacillus plantarum* for biotin determination in serum and urine. J Nutr Sci Vitaminol, 40: 491-498, 1994.

62. Ronald RE and Landen WO: Biotin, Vitamin Analysis for the Health and Food Sciences, 478-487, CRC Press, Boca Raton, (1998)

63. Ball GMF: Microbiological Methods for the Determination of the B-group Vitamins, Vitamins in Foods, 339-368, CRC Press, Boca Raton, (2005)

64. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25: 402-408, 2001.

65. Taniguchi A and Watanabe T: Transplacental transport and tissue distribution of biotin in mice at midgestation. Congenit Anom, 48: 57-62, 2008.

66. Zempleni J and Mock DM: Marginal biotin deficiency is teratogenic. Proc Soc Exp Biol Med, 223: 14-21, 2000.

67. Mock DM, Mock NI, Stewart CW, Laborde JB and Hansen DK: Marginal biotin deficiency is teratogenic in ICR mice. J Nutr, 133: 2519-2525, 2003.

68. Levin SW, Roecklein BA and Mukherjee AB: Intrauterine growth retardation caused by dietary biotin and thiamine deficiency in the rat. Res Exp Med, 185: 375-381, 1985.

69. Prasad PD, Wang H and Kekuda R et al.: Cloning and functional expression of a cDNA encoding a mammalian sodium-dependent vitamin transporter mediating the uptake of pantothenate, biotin, and lipoate. J Biol Chem, 273: 7501-7506, 1998.

70. Pacheco-Alvarez D, Solorzano-Vargas RS, Gonzalez-Noriega A, Michalak C, Zempleni J and Leon-Del-Rio A: Biotin availability regulates expression of the sodium-dependent multivitamin transporter and the rate of biotin uptake in HepG2 cells. Mol Genet Metab, 85: 301–307, 2005.

71. Vlasova TI, Stratton SL, Wells AM, Mock NI and Mock DM: Biotin deficiency reduces expression of SLC19A3, a potential biotin transporter, in leukocytes from human blood. J Nutr, 135: 42–47, 2005.

72. Reidling JC, Nabokina SM and Said HM: Molecular mechanisms involved in the adaptive regulation of human intestinal biotin uptake: a study of the hSMVT system. Gastrointest Liver Physiol, 292: 275–281, 2007.

73. Crisp SE, Griffin JB and White BR et al.: Biotin supply affects rates of cell proliferation, biotinylation of carboxylases and histones, and expression of the gene encoding the sodium-dependent multivitamin transporter in JAr choriocarcinoma cells. Eur J Nutr, 43: 23-31, 2004.

74. Xiao S, Hansen DK, Horsley ET, Tang YS, Khan RA, Stabler SP, Jayaram HN and Antony AC: Maternal folate deficiency results in selective upregulation of folate receptors and heterogeneous nuclear ribonucleoprotein-E1 associated with multiple subtle aberrations in fetal tissues. Birth Defects Res A Clin Mol Teratol, 73: 6-28, 2005.

75. Kim HW, Choi YJ, Kim KN, Tamura T and Chang N: Effect of paternal folate deficiency on placental folate content and folate receptor α expression in rats, Nutr Res Pract, 5: 112-116, 2011.

76. Fowden AL, Coan PM, Angiolini E, Burton GJ, Constancia M: Imprinted genes and the epigenetic regulation of placental phenotype. Prog Biophys Mol Biol, 106: 281-288, 2011.

77. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116: 281-297, 2004.

78. Bao B, Rodriguez-Melendez R, Wijeratne SS and Zempleni J: Biotin regulates the expression of holocarboxylase synthetase in the miR-539 pathway in HEK-293 cells. J Nutr, 140: 1546-1551, 2010.

79. Rodríguez-Meléndez R, Cano S, Méndez ST and Velázquez A: Biotin regulates the genetic expression of holocarboxylase synthetase and mitochondrial carboxylases in rats. J Nutr, 131: 1909-1913, 2001.

80. Gralla M, Camporeale G and Zempleni J: Holocarboxylase synthetase regulates expression of biotin transporters by chromatin remodeling events at the SMVT locus. J Nutr Biochem, 19: 400-408, 2008.

81. Sealey WM, Stratton SL, Mock DM and Hansen DK: Marginal maternal biotin deficiency in CD-1 mice reduces fetal mass of biotin-dependent carboxylases. J Nutr, 135: 973–977, 2005.

82. Dakshinamurti K, Modi VV and Mistry SP: Biotin deficiency produces hyperglycemia. Proc Soc Exp Biol Med, 127: 396-400, 1968.

83. Maebashi M, Makino Y, Furukawa Y, Ohinata K, Kimura S and Takao S: Therapeutic evaluation of the effect of biotin on hyperglycemia in patients with non-insulin diabetes mellitus. J Clin Biochem Nutr, 14: 211-218, 1993.

84. Nagamine T, Saito S, Yamada S, Arai T, Takehara K and Fukui T: Biotinidase activity in patients with liver disease. Scand J Gastroenterol, 28: 899-906, 1993.

85. Arbuckle HA and Morelli J: Holocarboxylase synthetase deficiency presenting as ichthyosis. Pediatr Dermatol, 23: 142-144, 2006.

86. Grunewald S, Champion MP, Leonard JV, Schaper J and Morris AA: Biotinidase deficiency: a leukoencephalopathy. Neuropediatrics, 35: 211-216, 2004.

87. Mikati MA, Zalloua P, Karam P, Habbal MZ and Rahi AC: Novel mutation causing partial biotinidase deficiency in a Syrian boy with infantile spasms and retardation. J Child Neurol, 21: 978-981, 2006.

88. Paul P K, Duttagupta P N and Agarwal HC: Effects of an acute dose of biotin on the reproductive organs of female rats. Curr Sci, 42: 206-208, 1973.

89. Paul PK, Duttagupta PN and Agarwal HC: Antifertility effect of biotin and its amelioration by estrogen in the female rat. Curr Sci, 42: 613-615, 1973.

90. Reeves PG: Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr, 127: 838-841, 1997.

91. Peters DM, Griffin JB, Stanley JS, Beck MM and Zempleni J: Exposure to UV light causes increased biotinylation of histones in Jurkat cells. Am J Physiol Cell Physiol, 283: 878-884, 2002.

92. Stanley JS, Griffin JB and Zempleni J: Biotinylation of histones in human cells: effects of cell proliferation. Eur J Biochem, 268: 5424-5429, 2001.

93. Manthey KC, Griffin JB and Zempleni J: Biotin supply affects expression of biotin transporters biotinylation of carboxylases and metabolism of interleukin-2 in Jurkat cells. J Nutr, 132: 887-892, 2002.

94. Scheerger SB and Zempleni J: Expression of oncogenes depends on biotin in human small cell lung cancer cells. Int J Vitam Nutr Res, 73: 461-467, 2003.

95. Lewis B, Rathman S and McMahon R: Dietary biotin intake modulates the pool of free and

protein-bound biotin in rat liver. J Nutr, 131: 2310-2315, 2001.

96. Zempleni J and Mock DM: Bioavailability of biotin given orally to humans in pharmacologic doses. Am J Clin Nutr, 69: 504-508, 1999.

97. Mock DM and Mock NI: Serum concentrations of bisnorbiotin and biotin sulfoxide increase during both acute and chronic biotin supplementation. J Lab Clin Med, 129: 384-388, 1997.

98. Food and Nutrition Board Institute of Medicine, Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B_6, Folate Vitamin B_{12}, Pantothenic Acid, Biotin and Choline. National Academy Press, Washington DC, (1998).

99. Dakshinamutri K and Chauhan J: Biotin. Vitamin Horm, 45: 337-384, 1989.

100. Paul PK, Duttagupta PN and Agarwal HC: Antifertility effects of biotin and its amelioration by estrogen in the female rat. Curr Sci, 42: 613-615, 1973.

101. Sawamura H, Fukuwatari T and Shibata K: Effects of excess biotin administration on the growth and university excretion of water-soluble vitamins in young rats. Biosci Biotechnol Biochem, 71: 2977-2984, 2007.

102. Paul PK and Duttagupta PN: The effect of an acute dose of biotin at the pre-implantation stage and its relation with female sex steroids in the rat. J Nutr Sci Vitaminol (Tokyo), 21: 89-101, 1975.

103. Paul PK and Duttagupta PN: The effect of an acute dose of biotin at a-post-implantation stage and its relation with female sex steroids in the rat. J Nutr Sci Vitaminol (Tokyo), 22: 181-186, 1976.

104. Mori A: The difference in sperm-morphology in different strains of mice. Tohoku J Agr Res, 12: 107-118, 1961.

105. Wyrobek AJ, Gorden LA, Burkhart JG, Francis MW, Kapp RW, Jr, Latz G, Malling HV, Topham JC and Whorton MD: An evaluation of human sperm as indicators of chemically induced alterations of spermatogenic function. Mutat Res, 115: 73-148, 1983.

106. Watanabe T and Endo A: Effects of selenium deficiency on sperm morphology and spermatocyte chromosomes in mice. Mutat Res, 262: 93-99, 1991.

107. Brown BW: A review of nutritional influences on reproduction in boars, bulls and rams. Reprod Nutr Dev, 34: 89-114, 1994.

108. Kaur P and Bansal MP: Effect of oxidative stress on the spermatogenic process and hsp70 expressions in mice testes. Indian J Biochem Biophys, 40: 246-251, 2003.

109. Webb EC, Dombo MH and Roets M: Seasonal variation in semen quality of Gorno Altai cashmere goats and South African indigenous goats. S Afr J Anim Sci, 34: 240-243, 2004.

110. Kawata T, Takeda T, Morimoto F, Fujimoto N, Tanaka N, Yamada K, Wada M, Tadokoro T and Maekawa A: Effects of vitamin B_{12}-deficiency on testes tissue in rats. J Nutr Sci Vitaminol (Tokyo), 38: 305-316, 1992.

111. Kawata T, Tamiki A, Tashiro A, Suga K, Kamioka S, Yamada K, Wada M, Tanaka N, Tadokoro T and Maekawa A: Effect of vitamin B_{12}-deficiency on testicular tissue in rats fed by pair-feeding. Int J Vitam Nutr Res, 67: 17-21, 1997.

112. Watanabe T, Ohkawa K, Kasai S, Ebara S, Nakano Y and Watanabe Y: The effects of dietary vitamin B_{12} deficiency on sperm maturation in developing and growing male rats. Congenit Anom (Kyoto), 43: 57-64, 2003.

113. Watanabe T, Ebara S, Kimura S, Maeda K, Watanabe Y, Watanabe H, Kasai S and Nakano Y: Maternal vitamin B_{12} deficiency affects spermatogenesis at the embryonic and immature stages

in rats. Congenit Anom (Kyoto), 47: 9-15, 2007.

114. Appling DR and Chytil F: Evidence of a role for retinoic acid (vitamin A-acid) in the maintenance of testosterone production in male rats. Endocrinology, 108: 2120-2124, 1981.
115. Van Pelt AM and de Rooij DG: Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells. Endocrinology, 128: 697-704, 1991.
116. Báez-Saldaña A, Camacho-Arroyo I and Espinosa-Aguirre JJ: Biotin deficiency and biotin excess: effects on the female reproduction. Steroids, 74: 863-869, 2009.
117. Paulose CS, Thliveris T, Viswanathan M and Dakshinamurti K: Testicular function in biotin-deficient adult rats. Horm Metab Res, 21: 661-665, 1989.
118. Veeramachaneni DN, Palmer JS, Amann RP, Kane CM, Higuchi TT and Pau KY: Disruption of sexual function, FSH secretion, and spermiogenesis in rabbits following developmental exposure to vinclozolin, a fungicide. Reproduction, 131: 805-816, 2006.
119. Song H, Liu J, An S, Nishino T, Hishikawa Y and Koji T: Immunohistochemical analysis of histone H3 modifications in germ cells during mouse spermatogenesis. Acta Histochem Cytochem, 44: 183-190, 2011.
120. Chew YC, West JT, Kratzer SJ, Ilvarsonn AM, Eissenberg JC, Dave BJ, Klinkebiel D, Christman JK and Zempleni J: Biotinylation of histonese represses transposable elements in human and mouse cells and cell lines and in Drosophila melanogaster. J Nutr, 138: 2316-2322, 2008.

Presented Paper

1. Sawamura H, Ishii Y, Shimada R, Yuasa M, Negoro M and Watanabe T: Low level of maternal biotin intake changes the expression of biotin transporter in dams and fetuses in mice. Int Natl J Anal Bio-Sci, 2014 (in press)
2. Watanabe T, Kioka M, Fukushima A, Morimoto M and Sawamura H: Biotin content table of select foods and biotin intake in Japanese. Int Natl J Anal Bio-Sci, 2014 (in press)
3. Sawamura H, Ikeda C, Shimada R, Yoshii Y and Watanabe T: Dietary intake of high-dose biotin inhibits spermatogenesis in young rats. Congenit Anom, 2014 (In press).
4. Koizumi M, Yoshii Y, Yuasa M, Sawamura H, Watanabe T, Yoshida E and Suzuki K: Effect of euglena on cedar pollen allergies in cry j1-sensitized mice. Nippon Shokuseikatsu Gakkaishi, 24: 171-176, 2014 (in Japanese).
5. Watanabe T, Masaki T, Yuasa M, Morimoto M and Sawamura H: Estimate of the dietary intake of biotin in infants prescribed special therapeutic infant formulas in Japan. Int J Anal Bio-Sci, 1: 60-70, 2013.
6. Watanabe T, Fukushima A, Matsumoto K, Sawamura H and Mizohata H: Estimated daily intake of biotin in hospital meals in Japan through dietary surveillance. Trace Nutr Res, 30: 64-73, 2013.
7. Yuasa M, Matsui T, Ando S, Ishii Y, Sawamura H, Ebara S and Watanabe T: Consumption of a low-carbohydrate and high-fat diet (the ketogenic diet) exaggerates biotin deficiency in mice.

Nutrition, 29: 1266-1270, 2013.

8. Watanabe T, Shimada R, Matsuyama A, Yuasa M, Sawamura H, Yoshida E and Suzuki K: Antitumor activity of the β-glucan paramylon from Euglena against preneoplastic colonic aberrant crypt foci in mice. Food Funct, 4: 1685-1690, 2013.
9. Yuasa M, Hashimoto T, Matsumoto K, Sawamura H, Matsui T, Kishimoto Y, Ishigami A and Watanabe T: Effects of diets and vitamin-enriched food on the nutritional state of victims in disaster shelters. Trace Nutrients Research, 30: 7−12, 2013 (in Japanese).
10. Matsumoto K, Yoshida M, Sawamura H and Watanabe T: Examination of the intake of micronutrients newly listed in standard tables of food composition in Japan 2010 ―iodine and selenium―. Trace Nutrients Research, 30: 27−30, 2013 (in Japanese).
11. Maekawa T, Kozai A, Iizuka A, Matsumoto K, Sawamura H, Hattori Y and Watanabe T: Comparative study on taste characteristics of Japanese and Chinese people. Trace Nutrients Research, 30: 44−47, 2013 (in Japanese).
12. Negoro M, Sawano M, Sawamura H, Ebara S and Watanabe T: Analysis of glucose-protein interaction using p-hydroxyacetophenone-Sepharose affinity resin-Glucose decreases alcohol dehydrogenase activity *in vitro*-. J Electrophoresis, 56: 19-24, 2012.
13. Hiura Y, Fukushima Y, Yuno M, Sawamura H, Kokubo Y et al: Validation of the association of genetic variants on chromosome 9p21 and 1q41 with myocardial infarction in a Japanese population. Circ J, 72: 1213-17, 2008.

14. Niwa Y, Hiura Y, Sawamura H and Iwai N: Inhalation of Exposure to Carbon Black Induces Inflammatory Response in Rats. Circ J, 72: 144-49, 2008.

15. Sawamura H, Fukuwatari T and Shibata K: Effects of Excess Biotin Administration on Growth and Urinary Excretion of Water-Soluble Vitamins in Young Rats. Biosci Biotechnol Biochem, 71: 2977-84, 2007.

Oral Presentation List

1. Molecular nutritional study on effects of biotin deficiency on fetal growth in the pregnant mice.

   Sawamura H, Ishii Y, Shimada R, Yuasa M, Negoro M, Watanabe T. The 54th Annual Meeting of the Japanese Teratology Society, Kanagawa, July, 2014.

2. A fundamental study on the kinetics of biotin in pregnant mice.

   Hiromi sawamura, Yoshie Ishii, Masahiro Yuasa, Munetaka Negoro, Toshiaki Watanabe. The 65th Annual Meeting of the Vitamin Society of Japan, Tokyo, June, 2013.

3. The study on food and nutrition management at the time of disaster –Effectiveness and application of vacuum cooking in terms of intaking vitamin C-.

   Hiromi Sawamura, Saeko Morii, Kaoru Sakamoto. The 59th Annual Meeting of the Japanese Society of Nutrition and Dietetics, Nagoya, September, 2012 (poster presentation).

4. The effect of biotin deficiency on biotin-related proteins in human embryonic palatal mesenchymal cells.

   Hiromi Sawamura, Toshiaki Watanabe. International Conference on Cofactors 03, Turku, Finland, July, 2011.

Acknowledgement

I wish to express my deep indebtedness to Prof. Katsumi Shibata and Prof. Tsutomu Fukuwatari, Department of Food Science and Nutrition, The University of Shiga prefecture, for giving a thorough guidance and encouragement throughout the preparation of this thesis.

I wish to express my deep gratitude to Prof. Toshiaki Watanabe, Department of Dietary Environment Analysis, School of Human Science and Environment, University of Hyogo and Prof. Munetaka Negoro, Department of Chemical and Biological Engineering, Ube National College of Technology, for many useful suggestions during this experiment.

I wish to express my thanks to the members of Food Environment Analysis Laboratory, School of Human Science and Environment, University of Hyogo for their technical support of this experiment.

I wish to express my gratitude to my mother and father for their understanding, encouragement and unconditional support.